PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/02, C07K 15/00, 15/04
C07K 15/28

A1

(11) International Publication Number: WO 91/15223

(43) International Publication Date: 17 October 1991 (17.10.91)

(21) International Application Number: PCT/US91/02136

(22) International Filing Date: 27 March 1991 (27.03.91)

(30) Priority data:
502,318 30 March 1990 (30.03.90) US

(71) Applicants: AMGEN INC. [US/US]; 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US). CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Drive, Ithaca, NY 14850 (US).

(72) Inventors: NARACHI, Michael, A. ; 2035 Poli Street, Ventura, CA 93001 (US). ARAKAWA, Tsutomu ; 4217 Minnecota Drive, Thousand Oaks, CA 91360 (US). NATHAN, Carl, F. ; 5 Edgewood Avenue, Larchmont, NY 10538 (US). SRIMAL, Subita ; 529 East 85th Street, New York, NY 10028 (US).

(74) Agent: MAZZA, Richard, J.; Amgen Inc., 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.

(54) Title: RESPIRATORY BURST SUPPRESSION FACTOR

(57) Abstract

Respiratory burst suppression factors are polypeptides having the ability to inhibit phagocyte activation or to reverse pre-existent activation of phagocytes. Methods for the preparation of these factors from cell culture medium are disclosed. Also disclosed are methods and compositions for the treatment of wounds, inflammatory disorders, and immune disorders using these factors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

RESPIRATORY BURST SUPPRESSION FACTOR

The present invention relates to novel factors which are polypeptides having the ability to inhibit the activation or reverse pre-existent activation of phagocytic cells (macrophages, and neutrophils) , to methods for preparing these factors, and to methods and compositions for the treatment of disorders using these factors.

Background of the Invention

Phagocytic cells (macrophages and neutrophils) are activated by various environmental stimuli and cytokines to kill tumor cells and microbial pathogens by releasing reactive oxygen intermediates (e.g. hydrogen peroxide) during a respiratory burst, or by releasing reactive nitrogen intermediates (e.g., nitric oxide).

Cytokines that enhance the cytotoxic and antimicrobial function of macrophages and neutrophils have been extensively studied. More recently, cytokines have been identified that block the induction of macrophage activation. These include transforming growth factor (TGF)-β, (Tsunawaki et al., Nature 334, 260 (1988)); interleukin (IL)-4 (Abramson et al., Clin. Res. 37, 825A (abstract) 1989) and calcitonin gene-related peptide (CGRP) (Nong et al., J. Immunol. 143, 45 (1989)).

The suppression of mouse peritoneal macrophage respiratory burst activity by tumor cell conditioned medium (TCM) has been described (Szurdo-Sudol et al., Fed. Proc. 41, 962 (abstract) 1982) and has been observed with medium conditioned by a variety of murine (Szuro-Sudol and Nathan, J. Exp. Med. 156, 945 (1982); Nelson et al., Aust. J. Exp. Biol. Med. 60,493 (1982))

and human tumor cell lines (Szuro-Sudol, Deactivation of Macrophages by Products of Other Cells, Ph.D. Thesis, Rockefeller University, 1985). Treatment of macrophages with TCM not only suppresses the production of reactive oxygen intermediates, but can also suppress the killing of some microbial pathogens.

Phagocytic cell activation and the release of toxic products during respiratory burst can be deleterious to normal cells and has been implicated in inflammatory responses (Gallin et al., Inflammation, Basic Principles and Clinical Correlates, Raven Press, New York, 1988). Thus, administering compositions that suppress phagocyte activation provides a means for reducing inflammatory conditions. European Patent Application 269408 hereby incorporated by reference, describes the use of TGF-β for the treatment of inflammatory disorders, but not as it pertains to the suppression of respiratory burst.

An object of this invention is to provide novel factors that inhibit the activation or reverse pre-existent activation of macrophages and neutrophils. A further object of the invention is the use of such novel factors for the treatment of disorders characterized by inflammation.

Summary of the Invention

The subject invention comprises novel polypeptides herein referred to as respiratory burst suppression factor, or RBSF having the ability to inhibit activation of phagocytes. These polypeptides have the property of inhibiting phagocyte respiratory burst, thereby inhibiting the release of toxic oxygen and nitrogen intermediates from phagocytes. These toxic intermediates provide a defense against diseases and

infections by killing tumor cells and microbial pathogens. However, prolonged release of oxygen and nitrogen intermediates can be deleterious to normal cells. Therefore, RBSF can be used to treat disorders resulting from prolonged phagocyte activation.

The subject invention provides a method for the production of RBSF comprising the steps of culturing a cell line which produces RBSF and isolating RBSF from the cell-conditioned culture medium. Any cell line producing RBSF can be used. For example, the cell line is P815, a mouse mastocytoma cell line, or K562 a human cell line derived from a patient having chronic myelogenous leukemia.

The subject invention also provides a method of purification of a polypeptide having the properties of RBSF wherein the polypeptide is distinct from TGF-β, IL-4, or CGRP. The method of purification of RBSF comprises the steps of gel filtration, ion exchange chromatography, and one or more steps of reverse phase high pressure liquid chromatography.

The subject invention further relates to pharmaceutically acceptable compositions of RBSF and to the methods of treatment involving the administration of a therapeutically effective dose of RBSF. RBSF is effective as an anti-inflammatory agent, a wound healing factor and an immunosuppressant.

Brief Description of the Drawings

Figure 1 shows the fractionation of RBSF by Sephacryl S-400 chromatography.

Figure 2 shows elution of RBSF from a Biogel P60 column.

Figure 3 shows elution of RBSF from a Mono Q anion exchange column.

Figure 4 shows the fractionation of RBSF by diphenyl reverse phase high pressure liquid chromatography (RPHPLC).

Figure 5 shows C4 reverse phase HPLC of RBSF using the bioactive peak from diphenyl C4 RPHPLC.

Figure 6 shows a second C4 RPHPLC fractionation of RBSF using the bioactive peak from the first C4 column.

Figure 7 shows inhibition by RBSF and TGF-β's of macrophage release of hydrogen peroxide.

Figure 8 shows inhibition by RBSF and TGF-β of macrophage release of nitrites induced by gamma-interferon and the combination of gamma-interferon and tumor necrosis factor-alpha.

Figure 9 shows stimulation of thymidine uptake by normal rat kidney fibroblasts in the presence of RBSF and TGF-β1.

Figure 10 shows inhibition of thymidine uptake by mink lung epithelial cells in the presence of RBSF and TGF-β1.

Figure 11 shows inhibition of thymidine uptake by concanavalin A-stimulated mouse splenocytes in the presence of RBSF and TGF-β1.

Figure 12 shows the inability of RBSF to compete with TGF-β1 for binding to fibroblasts and epithelial cells.

Figure 13 shows the suppression by human RBSF of H_2O_2 release by activated neutrophils.

Figure 14 shows a Biogel P60 fractionation of human RBSF.

Figure 15 shows an SDS-PAGE analysis of Biogel P60 fractions.

Figure 16 shows SP-5PW chromatography of human RBSF using active Biogel P60 fractions.

Figure 17 shows an SDS-PAGE analysis of SP-5PW fractions.

Detailed Description of the Invention

Respiratory burst suppression is defined herein as the suppression of release of reactive oxygen or nitrogen intermediates from activated phagocytes, for example, macrophages, and neutrophils. Previously, the suppression of reactive oxygen release was demonstrated by exposure of macrophages to tumor cell conditioned medium (TCM). The present invention provides for factors that are free from other components in TCM and have the ability to inhibit release of reactive oxygen intermediates by macrophages which have been activated by a variety of soluble and microbial triggering agents (see Example 2). In addition, it has also been found that the polypeptides of the present invention inhibit the release of reactive nitrogen intermediates triggered

by treatment of macrophages with γ-interferon or the combination of γ-interferon and TNF-α(see Example 3). Prolonged release of reactive nitrogen or oxygen intermediates is deleterious to normal cells and is suppressed by treatment with an effective amount of RBSF.

The biological activity of RBSF is not confined to the inhibition of macrophage activity. As described in Example 4, it has now been found that purified RBSF can affect the proliferation of nonmyeloid cell lines. RBSF stimulates the proliferation of kidney fibroblasts, and inhibits the proliferation of lung epithelial cells and T-lymphocytes. The stimulatory action of RBSF on fibroblasts is useful in accelerating wound healing while the inhibitory action on T lymphocytes is useful in suppressing immune responses.

A method for the production of RBSF is provided. This method comprises the steps of culturing a cell line which produces RBSF and isolating RBSF from the cell-conditioned medium. In embodiments of the invention, the cell line is for example P815, a mouse mastocytoma cell line, or K562, a human cell line derived from a patient having a chronic myelogenous leukemia.

Also provided by the present invention is a method for purifying RBSF from culture medium. The method comprises the steps of: subjecting RBSF-containing material to gel filtration chromatography (e.g., Sephacryl S400 or Biogel P60), subjecting RBSF-containing material to ion exchange chromatography (e.g., Mono Q), subjecting RBSF-containing material to diphenyl reverse phase high pressure liquid chromatography, and subjecting RBSF-containing material to two sequential fractionations on a reverse phase C4 column. Details in the use of this method are given in

Example 1. Throughout the procedure, the presence of RBSF was detected by the macrophage H_2O_2 release assay described in Example 1.

RBSF prepared by this method was purified over 6,000-fold starting from TCM (see Table 1). Purified RBSF is defined as a single, major band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of about 13,000 daltons. Alternatively, purified RBSF is defined as a single peak of activity on reverse phase high pressure liquid chromatography (see Fig. 6A), and as a single, major band on SDS-PAGE of about 13,000 daltons (see Fig. 6C).

It has been found that RBSF is a novel factor distinct from other factors such as TGF-β_1 that are known to suppress the respiratory burst of activated phagocytes. RBSF suppresses respiratory burst of phagocytic cells in the presence of phagocyte activating factor gamma interferon, whereas TGF-β does not suppress respiratory burst when gamm interferon is present. As described in Example 5, antibodies specifically binding TGF-β_1 fail to neutralize the ability of purified RBSF to suppress release of oxygen intermediates by macrophages. In addition, purified RBSF fails to compete with TGF-β_1 for binding to kidney fibroblasts and lung epithelial cells, which are cell lines which express TGF-β receptors. The molecular weight of RBSF distinguishes it from two other factors known to suppress macrophage activity, IL-4, which is about 20,000 daltons, and CGRP, which is about 4,500 daltons.

The factors of the present invention also encompass variations in the primary structure (i.e., amino acid sequence) of RBSF. Such variants include precursor forms of RBSF having additional amino acids at the amino terminus which are processed during secretion from host cells and allelic variants.

The invention also provides chemically modified forms of RBSF which exhibit increased solubility, stability and/or circulating half-life compared to unmodified RBSF. Such modifications include, for example, covalent linkage to polyethylene glycol (see U.S. Patent No. 4179337 hereby incorporated by reference).

The factors of the invention may be covalently attached to a radioactive (e.g., I-125) or nonradioactive (e.g., fluorescent dye reagent) reporter group to provide reagents useful in the detection or quantification of RBSF in solid tissues and fluid samples.

Also comprehended by the invention are pharmaceutical compositions comprising therapeutically effective amounts of RBSF together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Therapeutically effective amounts of RBSF (e.g., those that reduce the level of respiratory burst activity) in a pharmaceutical composition can be determined by the ordinary artisan, taking into account such variables as the half-life of RBSF preparations, route of administration and the clinical condition being treated. The RBSF compositions are administered by continous infusion, sustained release formulation, aerosol spray, or injection.

The invention provides for the use of RBSF alone or in combination with other therapy in the treatment of disorders characterized by inflammation resulting from excessive phagocyte activation. These disorders include, but are not limited to, the following: rheumatoid arthritis and other arthritic diseases including crystal induced inflammation (Gallin et al., Inflammation, Basic Principles and Clinical Correlates, Raven Press, New York, 1988, pp. 751-783);

asthma (Calhoun et al., Am. Rev. Respir. Dis. 135, 224A (abstract) 1987; Serhan et al., PNAS 81, 5335-5339 (1984)); emphysema (Mittman et al., 1986); systemic lupus erythematosus; adult respiratory distress syndrome (Gallin et al., supra, pp. 815-824); inflammatory bowel disease (Jorizzo et al., Arch. Intern. Med., 144, 738-740 (1984)) psoriasis (Cram et al., J.Am. Acad. Dermatol. 4, 1 (1981); and sarcoidosis (Robinson et al., J. Clin. Invest., 75, 1488-1495 (1985)).

In addition, the invention provides for the use of RBSF alone, or in combination with other therapy, as an antagonist to factors which, when administered, may promote deleterious effects associated with macrophage activation. In particular, RBSF may be used to counteract the effects of macrophage activation resulting from treatments with γ-interferon, the combination of γ-interferon and TNF, M-CSF, G-CSF or GM-CSF.

The invention encompasses the use of RBSF alone or in combination with other therapy for the treatment of wounds. Administration of RBSF at a wound accelerates tissue regeneration by stimulating the proliferation of fibroblasts and by suppressing the release of toxic molecules by macrophages at the wound site.

Also comprehended by the invention is the use of RBSF alone or in combination with other therapy to inhibit the proliferation of T lymphocytes, thereby acting as an immunosuppressant.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXAMPLE 1

Purification and Characterization of RBSF from the culture medium of P815 mouse mastocytoma cells

A. Bioassay for Respiratory Burst Suppression Activity.

Activated macrophages were washed from the peritoneal cavities of female CD1 mice (Charles River Laboratories, Wilmington, MA) with Eagle's minimal essential medium, α-variant (αMEM) (KC Biologicals, Lenexa, KS) 3-4 days after injection of 1 ml autoclaved 6% sodium caseinate (practical grade, Eastman Kodak Co., Rochester, NY) in normal saline, as described (de La Harpe et al., J. Immunol. Methods 78, 223 (1985)). The cells were centrifuged (1070 x g, 10 min, 4°C) and resuspended in αMEM containing 10% heat inactivated horse serum (HyClone Laboratories, Logan UT) with 100 U/ml penicillin and 100 µg/ml streptomycin. This medium will be referred to as M-10. $2x10^5$ cells in 25 µl were added to each well of 96- well plates (Costar Data Packaging, Cambridge, MA) containing test samples and horse serum such that the final volume was 135-150 ul and the final horse serum concentration was 10%. The cells were incubated overnight in 5% CO_2 at 37°C and assayed for H_2O_2 release as described (de la Harpe et al., supra, 1985). In brief, the plates were washed in saline and the adherent cells incubated in Krebs-Ringer phosphate buffer with glucose and 100 ng/ml phorbol myristate acetate as a trigger of the respiratory burst. The oxidation of the fluorescent indicator scopoletin by H_2O_2, catalyzed by horseradish peroxidase, was monitored fluorometrically for 45-90 min, the cell protein in the same wells was measured spectrophotometrically, and the nmol H_2O_2 released per mg adherent cell protein calculated. A range of volumes of RBSF-containing

fractions was tested; the volume of test sample required for 50% inhibition of H_2O_2 releasing capacity was determined by interpolation and defined as one unit. Units per ml of test sample were divided by mg protein per ml of test sample to give the specific activity of RBSF. Specific activity is about 10-fold higher when macrophages are incubated in RBSF for 48 rather than 24 h (Szuro-Sudol et al., supra 1983; Tsunawaki et al., supra, 1988); the shorter assay was used for convenience.

B. Tumor cell-conditioned medium (TCM).

P815 mouse mastocytoma cells (American Type Culture Collection, Rockville, MD) were cultured at an initial density of 1.5 x 10^6/ml in α-MEM. After 48 h, the medium was centrifuged (5225 x g, 30 min, 4°C) and the supernatant sterilized by filtration (0.45 μ, Millex-HA; Millipore, Bedford, MA) and stored at 4°C.

C. Concentration of RBSF activity from TCM.

Three methods were used alternatively.

(1) Solid ammonium sulfate was slowly added to TCM at 4°C with constant stirring, maintaining the pH at 7.0 with NaOH until 70% saturation (w/w). The suspension was stirred for 4-6 hrs. and the precipitate collected by centrifugation (12,100 x g, 30 min, 4°C). The pellet was dissolved in 20 mM phosphate buffer pH 7.2 and dialysed extensively against the same and finally against PBS (GIBCO, Grand Island, NY). The dialysate was centrifuged (12100 x g, 10 min, 4°C) and the supernatant filter-sterilized and stored at -20°C.

(2) TCM was concentrated by ultrafiltration on either YM-300, YM-100, YM-10, or YM-5 membranes (Amicon, Lexington, MA) under nitrogen at 4°C with slow stirring. Filtrate and retentate were both reserved. (3) TCM was

extracted with acid ethanol according to method of Roberts et al., Proc. Nat. Acad. Sci. USA 77, 3494 (1980)) with minor modifications. Briefly, 1 volume of TCM or the retentate obtained upon ultrafiltration through YM100 or YM5 membranes was mixed overnight at 4°C with 3 vol of 0.23 N HCl in 95% ethanol (in some experiments this contained 86 mg/l of phenylmethylsulfonylflouride [PMSF]). The acid-insoluble proteins were discarded after centrifugation (5225 x g, 10 min, 4°C) and the pH of the supernatant adjusted to 3-4 with ammonium hydroxide. The acid-soluble proteins were further concentrated by the addition of 2 volumes of anhydrous ether (Baker Laboratories, Phillipsburg, NJ) and 1 volume ethanol to 1 vol of supernatant. After 24 h at -20°C, precipitated proteins were collected by centrifugation (5225 x g, 20 min, 4°C) and the supernatant discarded. The precipitate was dissolved in 1 M acetic acid and dialysed against the same overnight at 4°C. Insoluble material was discarded and the supernatant lyophilized (Savant Instrument Co., Farmingdale, NY) and stored at -20°C.

D. Sephacryl S400 chromatography.

RBSF concentrated by methods (1) or (2) was fractionated on a 1.5x50 cm column of S-400 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS at 4°C at a flow rate of 10 ml/h. 4 ml fractions were collected and aliquots assayed for RBSF activity, protein content and polypeptide migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The same three analyses were conducted on fractions from each of the following chromatographic procedures.

RBSF activity was detected in both the void volume (molecular weight of 450 kilodaltons) and in late fractions (molecular weight of 12 to 25 kilodaltons (see Fig. 1)).

E. Biogel P60 chromatography.

Lyophilized RBSF extracted by method (3) was dissolved in 1 M acetic acid. The precipitate was removed by centrifugation and the supernatant further clarified by filtration (0.45 μ) and applied on a 1.5x100 cm column of Biogel P60 (Biorad Laboratories, Richmond, CA) equilibrated with 1 M acetic acid at room temperature. Six-ml fractions were eluted in 1 M acetic acid at a flow rate of 20ml/h. Most of the RBSF activity eluted in a broad peak between ubiquitin (8.7 kilodalton marker) and chymotrypsingen (25 kilodalton marker) as shown in Fig. 2A. SDS-PAGE under reducing conditions (Fig. 2B) detected very few polypeptides in the active fractions, with those less than 14 kilodaltons predominating.

F. Ion exchange chromatography.

Active fractions from the Biogel P60 column were pooled, dried and reconstituted at 10 mM histidine HCl buffer pH 6.0 containing 1 mM sodium azide and 0.5 mM PMSF (buffer A) and applied on a Mono Q column (Pharmacia, HR 5/5) equilibrated with buffer A flowing at 0.25 ml/min. Bound proteins were eluted in 0.5 ml fractions over 30 min with a linear gradient of NaCl increasing to 1 M in buffer A. The arrow in Figure 3 indicates the start of the gradient. RBSF activity was recovered in the flow-through and the first few included fractions as shown in Figure 3.

G. Reversed phase high pressure liquid chromatography (RPHPLC).

RBSF from the Mono Q step was applied to a 4.6 mm ID x 25 cm (5 μ pore) diphenyl RP column (Vydac, Hesparia, CA) equilibrated with 0.1% triflouracetic acid (TFA) (Pierce Chemical Co., Rockford, IL) at a flow rate of 1 ml/min. Bound proteins were eluted over 30 min in an increasing gradient of acetonitrile in 0.1% TFA. The elution profile was monitored at 214 and 280 nm and 1-ml fractions were collected, dried, and reconstituted in 0.1% TFA. RBSF activity eluted in a single fraction at 54 ± 3% acetonitrile (Fig. 4A). Analysis of this fraction on a reduced Phastsystem minigel (Pharmacia) revealed a single band at 11-13 kilodaltons (Fig. 4B). However, a large 10-25% gradient gel resolved this band into four fractions, and revealed a fifth at 55 kilodaltons (Fig. 4C).

The bioactive fraction was further purified by two sequential fractionations on a RP C4 column (4.6 x 50 mm, 6.5 μ pore) (Synchrom, Lafayette, IN) equilibrated with 0.1% TFA (phase A). Phase B was 100% acetonitrile in 0.1% TFA. Elution was monitored at 214 and 280 nm and 1 ml fractions were collected. In the first fractionation, RBSF activity eluted in a single peak at 50 ± 2% acetonitrile (Fig. 5). As revealed by SDS-PAGE, RBSF activity in fractions 49 and 50 corresponded to polypeptides in the 13 kilodaltons range (Fig. 5, inset). In a second C4 fractionation, the single peak of RBSF activity (Fig. 6A) corresponded to the sole absorbance peak (Fig. 6B). Silver-stained SDS-PAGE revealed one band at about 13 kilodaltons (Fig. 6C). Although not visible in the photograph, purified RBSF from this and two other preparations contained variable amounts of a 66 kilodalton protein

that has been identified as albumin by amino acid sequencing.

H. Electrophoresis and immunoblotting.

SDS-PAGE (15x17x0.08 cm gels) was carried out by the method of Laemmli (21). The proteins were fixed in 30% methanol and 10% acetic acid and visualized by silver staining with minor modifications of the method of Morrissey (22). For immunoblots, the unfixed proteins, including pure porcine platelet TGF-β_1 (R&D Systems, Minneapolis, MN), pure recombinant human TGF-β_1 (Genentech, Inc., South San Francisco, CA), and pure rat CGRP (Peninsula Laboratories, Belmont, CA), were transferred to nitrocellulose (0.22 μm, Schleicher and Schuell, Keene, NH) according to the method of Towbin et al. (Proc. Natl. Acad. Sci. USA 76 4350 (1979)). The transfer buffer contained 0.1% SDS in 40 mM glycine, 25 mM Tris, 20% methanol pH 8.3. After transfer, proteins were visualised with amido black or Ponceau red and blocked overnight in blocking buffer (5% nonfat dry milk in 20 mM Tris-buffered saline, pH 7.5 containing 0.2% Tween 20) at 4°C. The blots were incubated with turkey anti-TGFβ_1 IgG, nonimmune turkey IgG (National Cancer Institute, Bethesda, MD), purified rabbit IgG antibody to ubiquitin and ubiquitin-protein conjugates (Medical College of Wisconsin, Milwaukee, WI), a rabbit antiserum and a mouse mAb to retroviral p15E (Duke University, Durham, NC), or nonimmune rabbit IgG for 2-3 h at 37°C. The blots were washed 3 times with blocking buffer and then incubated in rabbit IgG anti-turkey IgG (Zymed Laboratories, San Francisco, CA) or goat IgG anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) coupled to alkaline phosphatase. Immune complexes were visualised after reaction with indolyl phosphate and nitroblue

tetrazolium (United States Biochemical Co., Cleveland, OH) in veronal acetate buffer pH 9.6.

Each of the immunoblots were negative when RBSF samples were tested.

I. Measurement of protein concentration.

Protein was determined by the method of Lowry et al. (J.Biol. Chem. 193, 265 (1959)) with bovine serum albumin as the standard. The concentration of protein in RBSF fractions of highest purity was determined by amino acid analysis after hydrolysis in 6 N HCl.

J. Control of bacterial lipopolysaccharide (LPS) contamination.

The water used to prepare all reagents was purified by reverse osmosis and redistilled in glass shortly before use. Glassware was baked for 4 h at 180°C. In some cases, LPS was removed from chromatographic columns by treatment with 0.1 N NaOH (26). LPS contamination of RBSF fractions, culture media and treatment and assay reagents was monitored by a chromogenic limulus amebocyte lysate assay (Whittaker, MA Bioproducts, Walkersville, MD) with a sensitivity of ~10 pg/ml. Test samples containing >0.3 ng/ml LPS were discarded. Monitoring of lipopolysaccharide contamination is essential since LPS is also a potent suppressor of macrophage respiratory burst activity.

K. Summary of purification of RBSF.

A summary of the purification of RBSF is shown in Table 1.

TABLE 1

Summary of Purification of RBSF

Stage	Specific Activity (U/mg protein)[1]	Recovery (%)[2]	Fold Purification
TCM	9.4 ± 0.8 (7)	100	1.0
Extract[3]	222 ± 21 (4)	106 ± 11	23.7
Gel Filtration[4]	1008 ± 174 (4)	46 ± 8	108
Anion Exchange[5]	1860 (2)	45	198
RPHPLC (diphenyl)	6060 (2)	14	647
RPHPLC (C4)[6]	57,534 ± 3318 (3)[7]	2	6140

[1] Mean ± SEM for number of batches of TCM shown in parentheses. Seven batches were processed through gel filtration and 3 through RPHPLC. Results are shown for fewer fractionations in cases where RBSF activity was not measured in enough doses to permit assignment of units, or protein was not measured.

[2] With reference to TCM

[3] Acid-ethanol extraction and ether precipitation

[4] Biogel P60

[5] Mono Q

[6] Two fractionations in tandem

[7] Corrected for estimated 50% albumin contamination

EXAMPLE 2

Inhibition of macrophage release of reactive oxygen intermediates by RBSF and TGF-βs.

α-MEM supplemented with 10% horse serum (Hyclone Laboratories, Logan, UT) was conditioned by the murine P815 mastocytoma line as described in Example 1. For experiments with neutralization by anti-TGF-β antibodies, TCM was prepared without serum (to avoid an exogenous source of TGF-β) and was acidified, neutralized and dialyzed as described (Tsunawaki et al., supra 1989) to activate latent TGF-β-related molecules, then used as the retentate after filtration on a YM-5 membrane (nominal molecular wieght cut-off, 5 kilodaltons) (Amicon, Danvers, MA). Alternatively, as indicated, RBSF was purified from serum-containing TCM by a 7-step procedure: acid-ethanol extraction, ether precipitation, size exclusion chromatography, anion exchange chromatography, and 3 steps of reverse phase chromatography. For receptor binding studies, RBSF was purified only through step 5. Purification was monitored by the macrophage H_2O_2 release assay described below, in which 1 unit of RBSF is defined as the volume required to inhibit by 50% the phorbol ester-induced release of H_2O_2 by in vivo-activated macrophages after a 24-h incubation in a final volume of 0.135-0.150 ml. This corresponds to 6.7-7.4 U/ml. As purified, 1 unit corresponds to ~17.4 ng. The apparent potency of RBSF is increased 10-fold by extending the bioassay to 48 h (Szuro-Sudol & Nathan, supra 1982). Human TGF-β_1 (50 μg/ml), TGF-β_2 (20 μg/ml) and TGF-β_3 (30 μg/ml) were recombinant proteins purified to homogeneity from Chinese hamster ovary cells (Graycar et al., Mol. Endocrinol. 3, 1977 (1989)). Unless indicated otherwise, statements regarding relative

potency of cytokines are based on molar concentrations resulting in 50% of the maximal inhibition or stimulation.

The fluorescent microtest plate assay for caseinate-activated mouse peritoneal macrophage H_2O_2 release has been detailed (de La Harpe et al., supra 1985). Test cytokines were added to peritoneal cell cultures in α-MEM with 10% horse serum for 24 h. The adherent cell monolayers were then washed and placed in Krebs'-Ringer's phosphate buffered saline containing glucose, sodium azide to inhibit catalase, the fluorescent indicator scopoletin, horseradish peroxidase to catalyze oxidation of scopoletin by H_2O_2, and phorbol myristate acetate to trigger the respiratory burst. Loss of fluorescence of scopoletin was recorded over 1-3 h, following which the amount of cell protein adherent in each well at the start of the assay was measured as described, without removing the assay medium. Results are expressed as nmol H2O2 per mg adherent cell protein per unit time. The suppression of macrophage H_2O_2 release by RBSF is shown in Fig 7B. The suppression of H_2O_2 release by added TGF-β, TGF-β_2 or TGF-β_3 is shown in Fig. 7A for comparison.

EXAMPLE 3

Inhibition of macrophage release of reactive nitrogen intermediates by RBSF and TGF-βs.

RBSF was purified from P815 mastocytoma cell conditioned medium by the procedures described in Example 1. Its specific activity was 5.7×10^4 units/mg protein. A unit of RBSF is defined as that amount of RBSF in a final culture volume of 0.15 ml which causes 50% suppression of macrophage H_2O_2 releasing capacity

after a 24-h incubation. One unit of RBSF per well is equivalent to 6.7 units/ml. The potency of RBSF in the assay for suppression of macrophage H_2O_2 releasing capacity increases by a factor of ~10 when the incubation with macrophages is extended to 48 h.

Murine interferon-γ (5.2×10^7 units/mg protein) and human TNF-α (4×10^7 units/mg protein) were from Genentech, Inc. (South San Francisco, CA). Human TGF-β1 (50 µg/ml) (Derynck et al., Nature 31b, 701 (1985)), human TGF-β2 (20 µg/ml) (Martin et al., EMBO J. 6, 3673 (1987)) and human TGF-β3 (30 µg/ml) (Derynck et al. EMBO J. 7, (1988)) were secreted from Chinese hamster ovary cells transfected with expression vectors using the cytomegalovirus promoter. They were purified to apparent homogeneity (Graycar et al. supra, 1989). Murine IL-4 (10^8 units/mg protein) was from Immunex, Seattle, WA.

The level of endotoxin (LPS) contamination was estimated with a chromogenic limulus amebocyte lysate assay as described in Example 1. All the cytokines at the dilutions tested contained less than 20 pg/ml of contaminating LPS, except IL-4, in which LPS was ~70 pg/ml at the highest concentration tested.

LPS prepared by phenol extraction from *Escherichia coli* 0111:B_4 or *Salmonella minnesota* (wild type) was from List Biologicals Laboratories, Campbell, CA.

Macrophage culture. Female CD1 mice (8- to 12-wks old) were from the Charles River Breeding Laboratories (Wilmington, MA), and were maintained in the Research Animal Resource Center at Cornell University Medical College. Macrophages were washed from the peritoneal cavity 4 days after i.p. injection of 2 ml 4% thioglycollate broth as described (Ding, et al., J. Immunol. 141, 2407). Adherent monolayers were

obtained by plating 1 x 10^5 cells/well in 96-well plates (Costar Data Packaging, Cambridge, MA) in complete medium, consisting of αMEM with 8% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 50 U/ml of penicillin, and 50 µg/ml of streptomycin. The LPS level in complete medium was <20 pg/ml. After incubation for 2 h at 37°C in 5% CO2/95% air, the nonadherent cells were removed by aspiration and freshly prepared complete medium with cytokines was added.

Assays for NO_2^- and NO_3^-. Accumulation of nitrite in the medium was measured by an automated colorimetric assay based on the Griess reaction (Green et al., Proc. Natl. Acad. Sci. USA 78, 7764 (1981)). Briefly, samples were reacted with 1% sulfanilamide, 0.1% naphthylethylene-diamine dihydrochloride, and 2.5% H_3PO_4 at room temperature for 10 min, and NO_2^- concentration was determined by absorbance at 550 nm in comparison with sodium nitrite standards. Where indicated, nitrate in the sample was reduced to nitrite as described (Green et al., supra, 1981). Data are expressed as nmols nitrite, or nmols nitrite plus nitrate, per 10^5 cells originally plated. In all experiments, nitrite content in wells containing medium without cells was also measured, and subtracted. Suppression of γ-interferon induced nitrite release by RBSF and TGF-β, TGF-β_2, TGF-β_3, is shown in Fig. 8A. Suppression of γ-interferon plus TNF-α induced nitrite release is shown in Fig. 8B.

EXAMPLE 4

Activity of RBSF on nonmyeloid cell lines

Cytokines were prepared as described in Example 2.

A. Stimulation of fibroblast proliferation by RBSF.

The mitogenic assay on NRK49-F cells was essentially as described (Assoian et al., J. Biol. Chem. 258, 7155 (1983)). 4 x 10^3 cells in α-MEM with 10% bovine calf serum (BCS;Hy-Clone) were incubated overnight in 6-mm-diam flat-bottom wells in 96-well plates (Costar Data Packaging, Cambridge, MA). The next day, the cells were washed twice with α-MEM and incubated for 2 d in α-MEM with 0.2% BCS at 37°C in 5% CO2. The growth-arrested cells were then incubated with cytokines for 24 h. Four h before the end of the incubation, 1 μCi thymidine-methyl-^{3}H (2Ci/mmol, Dupont-New England Nuclear, Wilmington, DE) was added (final volume, 200 μl) (the same amount of the same 3H-thymidine preparation was used for the assays described below). For liquid scintillation counting, the cells were washed with α-MEM and the DNA was precipitated with cold 10% TCA, washed with 90% ethanol and solubilized in 0.5 N sodium hydroxide.

The stimulation of ^{3}H-thymidine uptake into rat kidney fibroblasts by RBSF and by TGF-β_1, is shown in Figure 9.

B. Inhibition of epithelial cell proliferation by RBSF.

Mink epithelial cells (American Type Culture Collection CCL64) were used as described (Tucker et al., Science 236, 705 (1984)). 3x10^4 cells were cultured in 200 μl of a-MEM with 1% BCS overnight at 37°C in 5% CO_2. The cells were washed, then incubated with cytokines in

a total volume of 150 µl for 24 h. ^{3}H-Thymidine was added during the last 4-8 h of incubation and DNA prepared for liquid scintillation counting as above.

The inhibition of ^{3}H-thymidine uptake into mink epithelial cells by RBSF and TGF-β, is shown in Figure 10.

c. Inhibition of T cell proliferation.

BALB/c or C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were sacrificed by cervical dislocation and the spleens removed aseptically. Single-cell suspensions were prepared as described (Kehrl et al., J. Exp. Med. 163, 1037 (1986) and the cells suspended in a α-MEM with 10% BCS. $5x10^{5}$ cells were cultured in 6-mm-diam flat-bottom wells in 96-well plates with 4 µg of concanavalin A (Sigma Chemical Co., St. Louis, MO) in a total volume of 250 µl for 24 h, followed by one wash with 0.3 M methyl-a-D-mannoside (Sigma) in a-MEM. Cytokines were added in α-MEM with 1% BCS and the plates incubated for 72 h at 37°C in 5% CO_2. ^{3}H-Thymidine was added for the last 24 h, after which the samples were processed with a semi-automated cell harvester (Cambridge, Technology Inc., Watertown, MA) for liquid scintillation counting.

Inhibition of ^{3}H-thymidine uptake into mouse spleen cells stimulated with the selective T-lymphocyte mitogen concanavalin A by RBSF and TGF-β, is shown in Fig. 11B and 11A, respectively.

EXAMPLE 5

Relatedness of RBSF and TGF-βs.

A. Inability of Anti-TGF-β antibody to neutralize purified RBSF

Preparation of cytokines and antibodies and assays for macrophage H_2O_2 release are described in Example 1. RBSF purified as described in Example 1 was incubated with turkey anti-TGF-β antibody which specifically binds TGF-β_1. Table 2 shows that the antibody completely neutralized the activity of TGF-β_1, on activated macrophages, but had no effect on the activity of RBSF on activated macrophages.

TABLE 2

Inability of Anti-TGF-β Antibody
to Neutralize Purified RBSF

Cytokine[1]	Antibody	H_2O_2 Release[2]
--	--	413 ± 41
TGF-β1[3]	pre-immune Ig[4]	93 ± 7
	immune IgG[5]	403 ± 10
--	--	481 ± 93
RBSF[6]	--	333 ± 22
	pre-immune IgG	276 ± 12
	immune IgG	316 ± 21

[1]Macrophages were incubated in M10 alone or with the indicated cytokines for 24 h, washed, and challenged with phorbol myristate acetate.

[2]Nmol/mg protein in 60 min (mean ± SEM of triplicates).

[3]3.5 ng/ml of pure TGF-β1 from porcine platelets.

[4]Purified by ammonium sulfate precipitation from turkey serum and included the bioassay at 40 µg/ml.

[5]Purified turkey IgG no. 121, included in the bioassay at 64 µg/ml, enough to neutralize completely 10 ng/ml TGF-β in this assay.

[6]Final preparation from second C4 column, diluted to 0.26 ng/ml to give partial inhibition to increase the chance of neutralizing inhibitory activity.

B. Antibody neutralization studies.

Anti-TGF-β antisera were raised in turkeys and rabbits as described (Danielpour et al., J. Cell Physiol. 138, 79 (1989)). Transiently acidified TCM, transiently acidified control medium, and TGF-β1 or TGF-β2 diluted in control medium were incubated with rabbit pre-immune or immune sera (final dilution 1:250) for 30 min at 37°C, followed by the addition of 25 µl of a 50% suspension of Protein-A agarose (Pharmacia Fine Chemicals, Piscataway, NJ). The mixture was further incubated for 30 min with intermittent shaking, then centrifuged. The supernatant was sterilized by filtration and assayed for residual inhibitory activity in the macrophage H_2O_2 release assay described below. Alternatively, test samples were incubated with turkey antisera or nonimmune sera (1:125) for 30 min at 37°C and added directly to macrophage cultures.

Table 3 demonstrates that antibodies to TGF-β_1, and/or TGF-β_2 have no effect on the activity of RBSF on activated macrophages. RBSF in this experiment is assayed in TCM and is not purified.

TABLE 3

Inability of Anti-TGF-β Antisera to Neutralize or Immunodeplete RBSF

Antibody†	Sample			
	Control Medium (100 µl)	TCM* (100 µl)	TGF-β1 (1 ng/ml)	TGF-β2 (8 ng/ml)
	H_2O_2, nmol/mg cell protein/180 min‡			
None	458 ± 42Δ	47 ± 7	47 ± 2	44±6
Rab preimmunet†	403 ± 14	43 ± 6	24 ± 7	ND**
Tur preimmunet†	463 ± 16	50 ± 11	18 ± 1	ND
α-TGF-β1 rab Har	572 ± 31	10 ± 5	211 ± 8	ND
α-TGF-β1 tur 367	427± 43	62 ± 7	246 ± 1	ND
α-TGF-β1 tur 121	427 ± 43	74 ± 122	601 ± 42	ND
α-TGF-β2 rab Sam	315 ± 16	28 ± 4	ND	512 ± 34
α-TGF-β2 tur 32	485 ± 20	33 ± 1	87± 14	379 ± 12

*To render TGF-β-like molecules more reactive with anitbodies, TCM and control medium were acidified, neutralized, and dialyzed.

†Antisera and control sera were used for immunodepletion at 1:250 (rabbit) or for neutralization at 1:125 (turkey).

ΔActivity in untreated control medium was 481 ± 14.

‡Results are means ± SEM from 3 experiments, each in triplicate.

††Pool of preimmune sera from each animal whose antiserum was used.

** Not determined.

C. Binding to TGF-β receptors.

Five ug of purified recombinant TGF-β_1 in protein-free vehicle was iodinated with carrier free ^{125}I (Amersham, Arlington Heights, IL) using chloramine-T (Sigma) as described (Frolik et al., 1984; Massague, 1987) to a specific activity of 133 μCi/μg, based on 60% recovery. Nonspecific binding was measured in the presence of 100- to 200-fold excess of nonradiolabelled TGF-β_1. Binding to NRK-49F and mink lung epithelial cells was carried out in 16-mm-diam wells in 24-well plates. Confluent monolayers were incubated in binding buffer (α-MEM with 0.1% bovine serum albumin in 25 mM Hepes, pH 7.5) for 2 h at 37°C and washed twice with binding buffer before addition of cytokines as potential competitors of binding of 100 pM ^{125}I TGF-β1 in a final volume of 375 μl. After 1 h at 37°, the plates were placed on ice. The monolayers were washed 4 times with cold binding buffer and solubilized in 1% Triton X-100, 10% glycerol and 25 mM Hepes, pH 7.5 for γ-counting. Binding to the pituitary cell line GH_3 (American Type Culture Collection) was tested as described (Cheifetz et al., J.Biol. Chem 263, 17225 (1988). Based on the saturable, specific binding of chloramine T-radioiodinated TGF-β_1 to NRK-49F cells (Fig. 12, inset), a concentration of 100 pM ^{125}I-TGF-β_1 was chosen to study the ability of other cytokines to compete with its binding. On normal rat kidney fibroblasts (Fig. 12A and mink lung epithelial cells (Fig. 12B, TGF-β_2 and TGF-β_3 in 40- to 50-fold molar excess each decreased the binding of TGF-β_1 by 70-75%. In contrast, at concentrations that were bioactive on the same cell types, RBSF showed no dose-dependent inhibition of the binding of TGF-β_1.

EXAMPLE 6

Identification of a human cell line having respiratory burst suppression activity.

Four human cell lines were tested for their ability to suppress H_2O_2 release by activated macrophages. They were U937 (ATCC No. CRL-1593) from a patient having histiocytotic lymphoma, HL-60 (ATCC No. CCL-240) from a patient having promyelocytic leukemia, THP-1 (ATCC No. TIB-202) from a patient having monocytic leukemia, and K-562 (ATCC No. CCL-243) from a patient having chronic myelogenous leukemia. Cell lines were tested and found to be negative for the presence of mycoplasma and viruses (MAP test) by Microbiological Associates (Rockville, MD).

TCM from human cell lines was prepared by culturing cells in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with L-glutamine to 2 mM and horse serum to 10%. After incubation at 37°C for four to five days, cells were pelleted by low speed centrifugation. TCM from each cell line was assayed for respiratory burst suppression and for the presence of LPS as described in Example 1. Of the four human cell lines tested, only TCM from K562 gave at least 50% inhibition of H_2O_2 release. TCM from the other cell lines tested had little effect on H_2O_2 release.

EXAMPLE 7

Suppression of respiratory burst activity in neutrophils.

Medium conditioned by P815 mouse mastocytoma cells and K562 human cells were prepared as described in

Example 6. Release of H_2O_2 bovine neutrophils which were untreated or treated with γ-interferon was assayed as a function of increasing amounts of TCM as described in Example 1. The suppression of H_2O_2 release from bovine neutrophils by human, but not mouse, TCM is shown in Figure 13. F250 refers to TCM from P815 cells.

EXAMPLE 8

Purification of RBSF from the culture medium of K562 human leukemia cell line.

A. Bioassay for RBSF activity.

RBSF activity was monitored during the purification as described in Example 1.

B. TCM.

K562, a human cell line from a patient having chronic myelogenous leukemia, was cultured at an intial density of 1 x 10^5/ml in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with L-glutamine to 2 mM and horse serum to 1%. Cells were incubated at 37°C for four to five days prior to harvesting.

C. Concentration of RBSF activity from TCM.

65 liters of TCM were concentrated by a Pellicon ultrafiltration apparatus to 3.2 liters and dialyzed against two changes of 40 liters of 1M acetic acid/40% ethanol at 4°C for 40 hours. The resulting preciptitated material was centrifuged and the supernatant containing RBSF activity was saved. The pellet from this centrifugation, which also exhibited significant RBSF activity, was taken up in 1M acetic acid containing 50% ethanol and re-extracted as

described above. The resulting supernatants were dialyzed against 40 liters of 1M of acetic acid. This concentrated material contained 8.1 g. of total protein.

D. Biogel P60 Chromatography.

200 mgs of protein from acetic acid/ethanol extraction were loaded onto a 1.95 liters of Biogel P60 column. The column was equilibrated in 1M acetic acid and proteins were eluted at a flow rate of about 1 ml/min. Fractions were collected and assayed for UV-absorbing material at 280 nm and for RBSF activity (Fig. 14), RBSF activity in this example is expressed as fluorescence magnitude, which is related to H_2O_2 release as described in Example 1.. Active fractions (about 275 ml) were pooled and concentrated by lyophilization. Fig. 15 shows an SDS-PAGE analysis of the Biogel P60 column fractions. Active fractions 80-96 were predominately polypeptides having a weight of less than about 25 kilodaltons.

For a larger scale purification, 1.75 g. of protein from acetic acid/ethanol extraction (about 20 to 50% of the total amount extracted) was loaded at one time onto a 13.6 liters bed volume Biogel P60 column. Four or five loadings were required to process the entire acetic acid/ethanol extract. The column was equilibrated in 1 M acetic acid and proteins were eluted at a flow rate of about 15 ml./min. 50 ml fractions were collected and 1 ml of each fraction was assayed for absorbance at 280 nm and RBSF activity. Active fractions were pooled (1.8 liter per run) and concentrated 50 to 100-fold by lyophilization or by filtration using a YM-5 membrane filter (Amicon, Danvers, MA) after adding 0.01% PEG-600 to prevent binding of RBSF to the filter.

E. Ion Exchange Chromatography.

Active fractions from the 1.9 liter Biogel P60 chromatography were dialyzed against 10 mM histidine, pH 6.0 and applied to a DEAE-5PW column (3.7 ml of resin), equivalent to MonoQ, equilibrated in the same buffer. As described in Example 1, RBSF activity was not retained on the column under these conditions. Bound proteins were eluted with a linear gradient of NaCl to 0 to 2M in the histidine buffer.

The flow-through material was applied to a SP-5PW column (3.7 ml of resin) under the same conditions as in the DEAE-5PW column and bound proteins were eluted in a similar NaCl gradient as in the DEAE column. Fractions were monitored for absorbance at 280 nm (Fig. 16) and were then pooled and concentrated prior to assay for RBSF activity. Pool-A contains flow-through material from the SP-5PW column, poolB contains fractions 1-4, pool-C contains fractions 5-6, pool-D contains fraction 8-9 and pool-E contains fractions 10-11. SDS-PAGE analysis of these pools is shown in Fig. 17. Pool-D and E both had RBSF activity, with pool-E having a higher level of activity. Pool-E is predominately a single polypeptide having a molecular weight of about 13 kilodaltons.

Based on the above observation, active pooled and concentrated fractions from the 13.6 liter. Biogel P60 chromatography were applied directly to an SP-5PW column under the conditions described above. A linear NaCl gradient from 0 to 0.4 M NaCl eluted the bulk of the 280 absorbing material that had bound to the column. Fractions collected during gradient elution are assayed for RBSF activity.

The material from pool-E (about 3 µg) is concentrated and applied to a diphenyl reverse phase column equilibrated in 0.1% TFA and eluted in an

increasing gradient of acetonitrile in 0.1% TFA as described in Example 1. Active fractions eluting from a dephenyl reverse phase column are then purified on a reverse phase C4 column equilibrated in 0.1% TFA (phase A). Phase B was 100% acetonitrile in 0.1% TFA. In both steps, fractions are assayed for UV absorbance and RBSF activity.

EXAMPLE 9

Amino acid sequences of human RBSF tryptic peptide

Approximately 3 µg of pool-E described in Example 7 was reduced with dithiothreitol and alkylated with iodoacetate to carboxymethylate cysteine residues. The modified protein was digested with trypsin for 14 hrs. and the tryptic fragments were chromatographed by reverse phase HPLC. Several major peptide fractions were detected. The sequence of fragment T-21 is:

$$A\text{-}G\text{-}L\text{-}H\text{-}S\text{-}P\text{-}T\text{-}P\text{-}\overset{(Q)}{(C)}\text{-}V$$

* * *

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED IS:

1. Purified respiratory burst suppression factor.

2. A factor according to Claim 1 which has a molecular weight of about 13,000 daltons as determined by SDS-PAGE.

3. A factor according to Claim 1 which has the ability to suppress phagocyte respiratory burst.

4. A factor according to Claim 1 having the ability to stimulate proliferation of fibroblasts.

5. A factor according to Claim 1 having the ability to inhibit proliferation of epithelial cells and T lymphocytes.

6. A factor according to Claim 1 which is covalently associated with a labelled compound that allows detection of the factor.

7. A factor according to Claim 1 which is murine respiratory burst suppression factor.

8. A factor according to Claim 1 which is human respiratory burst suppression factor.

9. An antibody specifically binding respiratory burst suppression factor.

10. An antibody according to Claim 9 which is monoclonal.

11. A method for the production of respiratory burst suppression factor comprising the steps of:

a) culturing a cell line producing respiratory burst suppression factor; and

b) isolating respiratory burst suppression factor from the culture medium of the cell line.

12. A method according to Claim 11 wherein the cell line of step (a) is K562 (A.T.C.C. No. CCL-243).

13. A method according to Claim 11 wherein step (b) comprises:

subjecting material containing respiratory burst suppression factor to gel filtration;

subjecting the gel filtered material to ion exchange chromatography; and

subjecting the ion exchange material to one or more cycles of reverse phase-high pressure liquid chromatography.

14. A pharmaceutical composition comprising a therapeutically effective amount of a factor according to Claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

15. A method for the treatment in a mammal of an inflammatory disorder comprising administering a therapeutically effective amount of a factor according to Claim 1.

16. A method according to Claim 15 wherein the inflammatory disorder results from an autoimmune disease.

17. A method according to Claim 16 wherein the autoimmune disease is rheumatoid arthritis or systemic lupus erythematosus.

18. A method according to Claim 15 wherein the inflammatory disorder results from an inflammation of lung and distal air passages.

19. A method according to Claim 18 wherein the inflammatory disorder is asthma, emphysema, sarcoidosis, or adult respiratory distress syndrome.

20. A method according to Claim 15 wherein the inflammatory disorder results from an inflammation of the skin or integument.

21. A method according to Claim 20 wherein the inflammatory disorder is psoriasis.

22. A method according to Claim 15 wherein the inflammatory disorder results from inflammatory bowel disease.

23. A method according to Claim 15 wherein the inflammatory disorder results from the administration of a macrophage activation factor.

24. A method according to Claim 23 wherein the macrophage activation factor is gamma-interferon or a combination of gamma-interferon and tumor necrosis factor-alpha.

25. A method for the treatment of a wound in a mammal comprising administering a therapeutically effective amount of a factor according to Claim 1.

26. A method for the suppression of an immune response in a mammal comprising administering a therapeutically effective amount of a factor according to Claim 1.

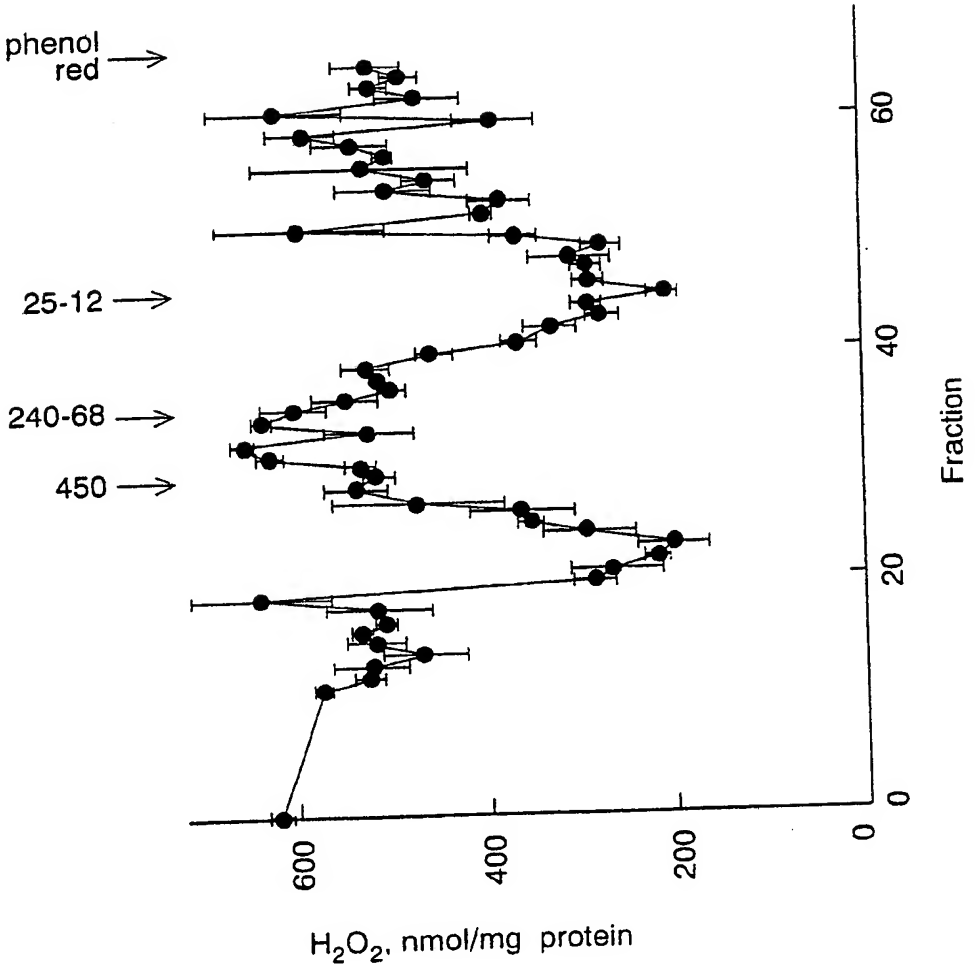

phenol red
25-12
240-68
450
0
20
40
60
Fraction
600
400
200
0
H_2O_2, nmol/mg protein

FIG. 1

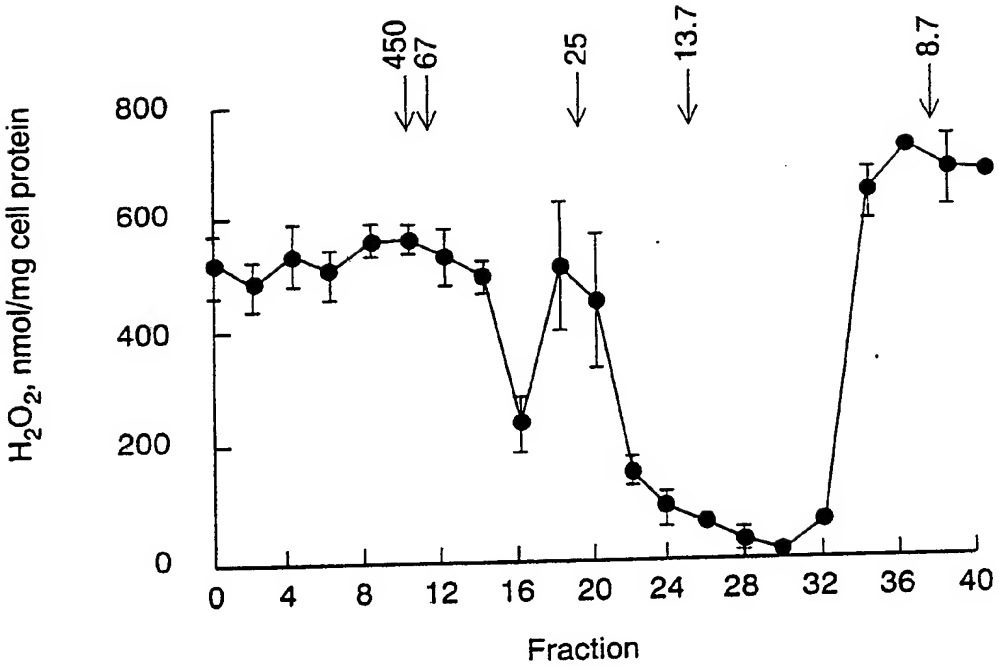
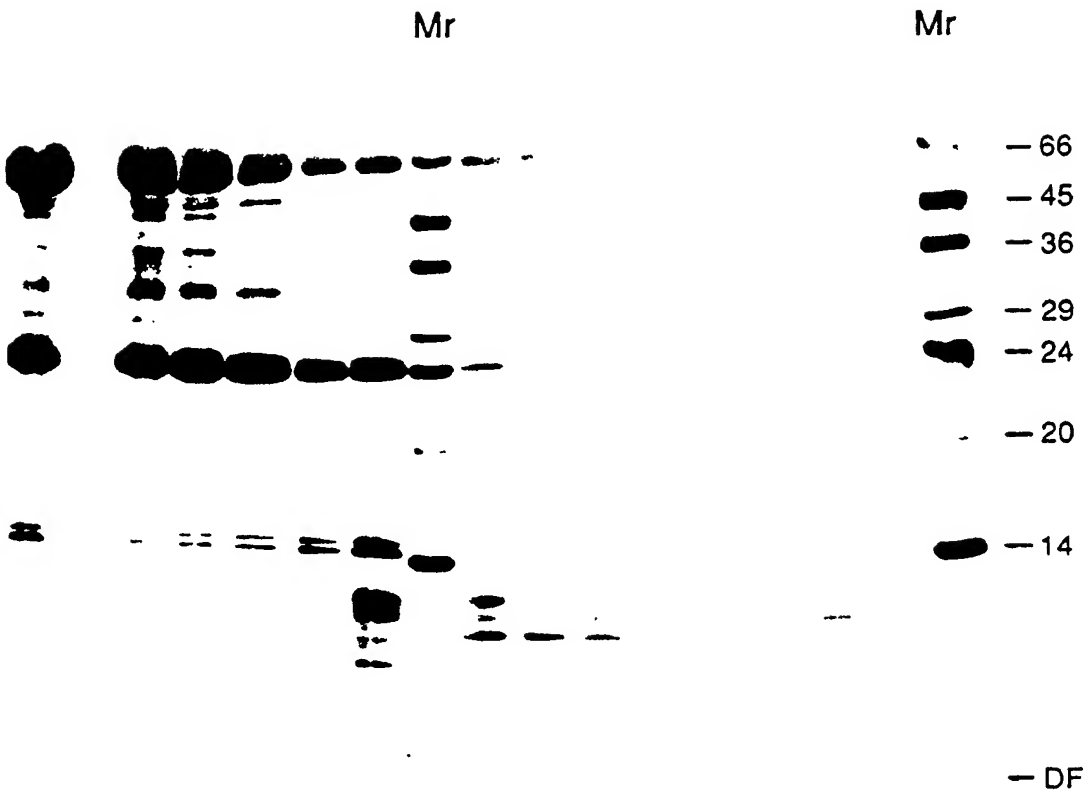

450
67
25
13.7
8.7
800
600
400
200
0
H_2O_2, nmol/mg cell protein
0
4
8
12
16
20
24
28
32
36
40
Fraction

FIG. 2A

FIG. 2B


Mr
Mr
66
45
36
29
24
20
14
DF
16 18 19 20 21 22 24 26 27 28 29 30 32 34
Fraction

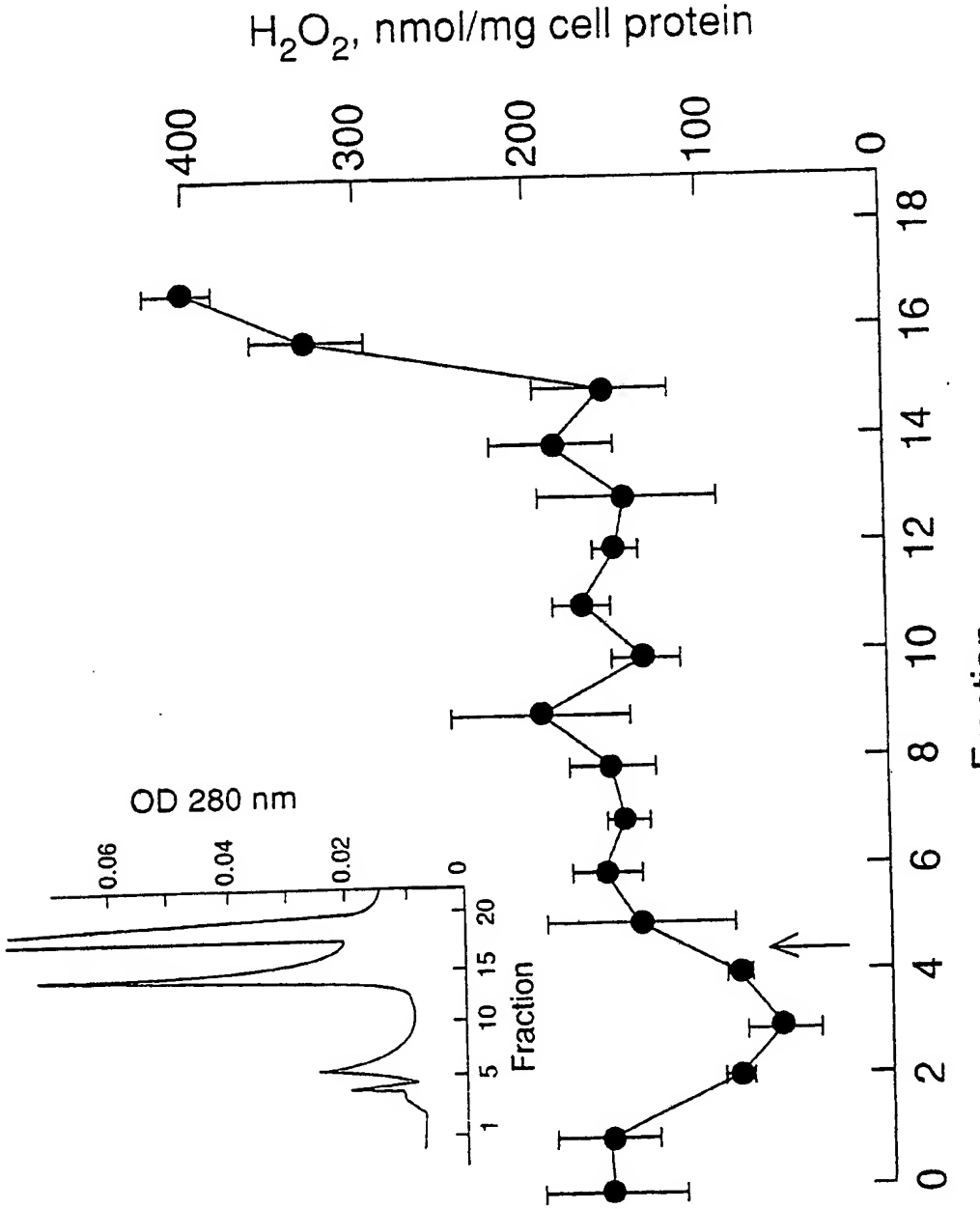

H_2O_2, nmol/mg cell protein
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
Fraction
OD 280 nm
0.06
0.04
0.02
0
1
5
10
15
20
Fraction

FIG. 3

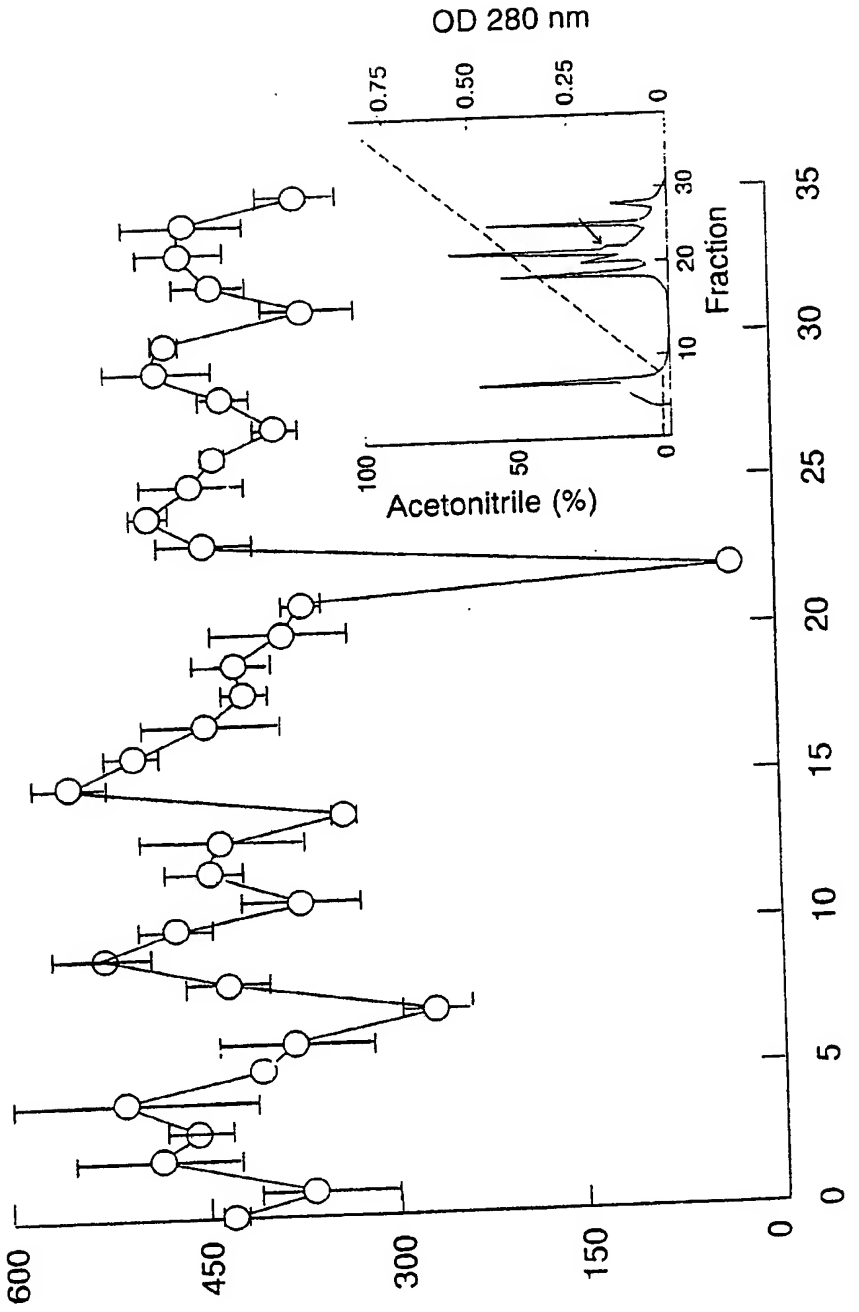

OD 280 nm
0.75
0.50
0.25
0
Acetonitrile (%)
100
50
0
Fraction
30
20
10
0
Fraction
35
30
25
20
15
10
5
0
600
450
300
150
0
H_2O_2 , nmol/mg cell protein

FIG. 4A

FIG. 4B

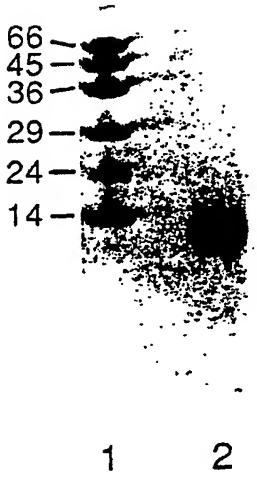


FIG. 4C

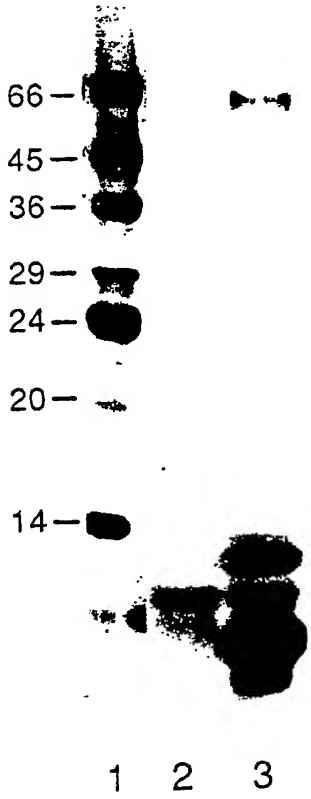


FIG. 5A

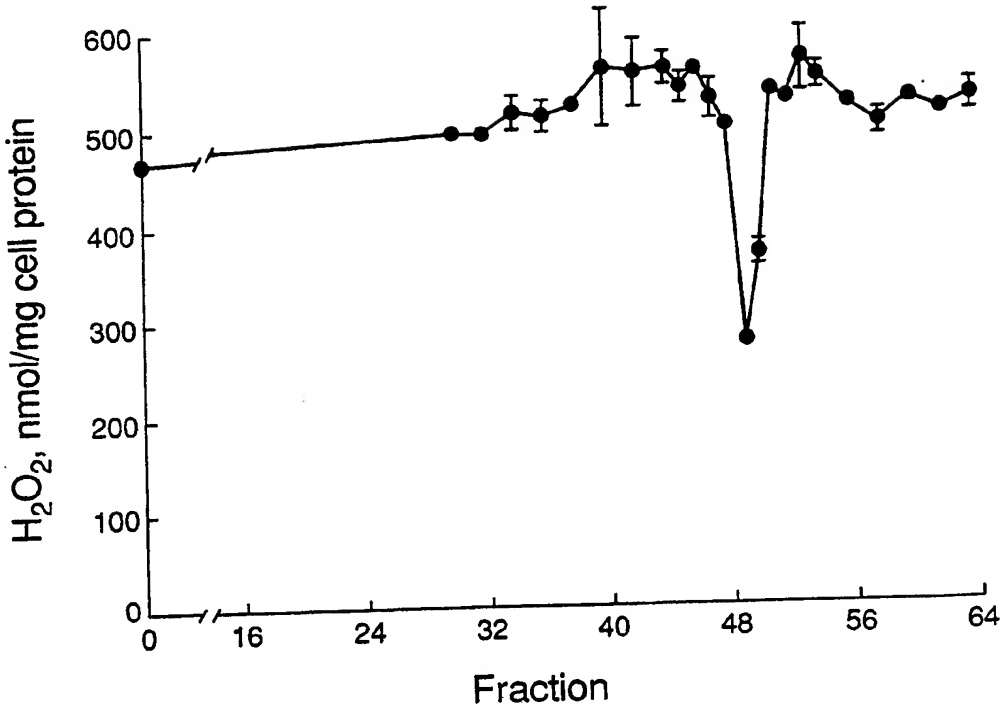
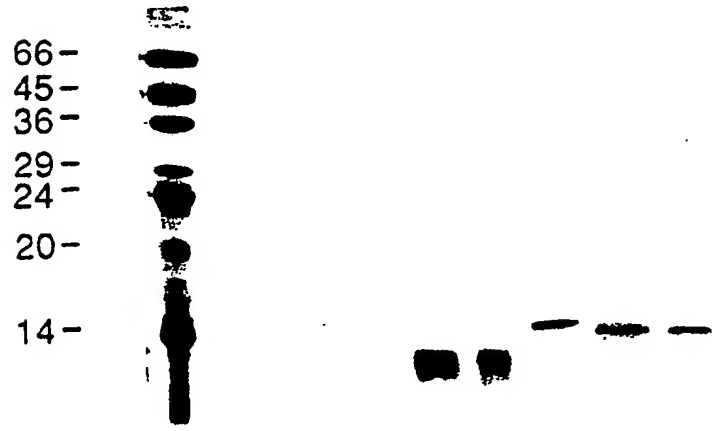


FIG. 5B



Mr 40 44 45 46 47 48 49 50 53 54

FIG.6A

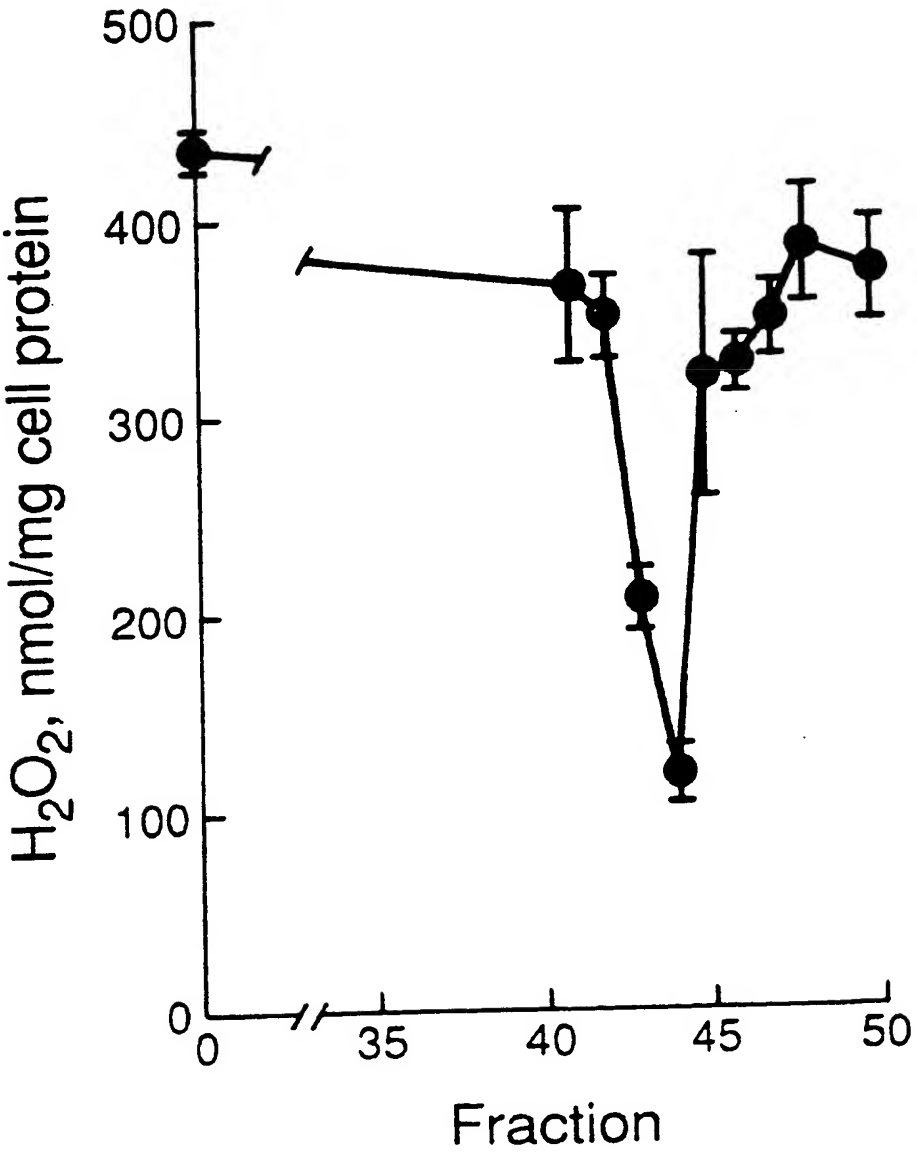
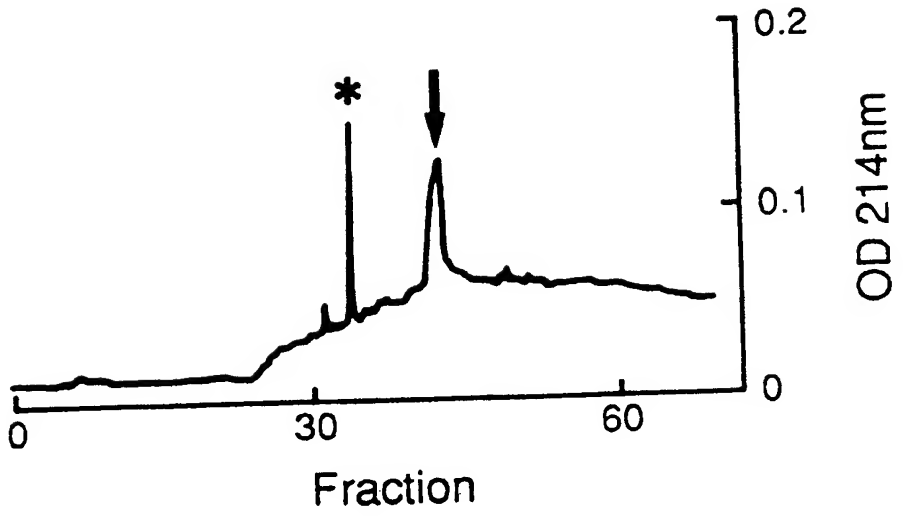

500
400
300
200
100
0
0
35
40
45
50
H_2O_2, nmol/mg cell protein
Fraction

FIG.6B



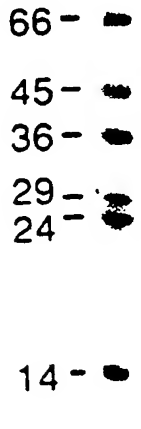


FIG.6C

FIG. 7B

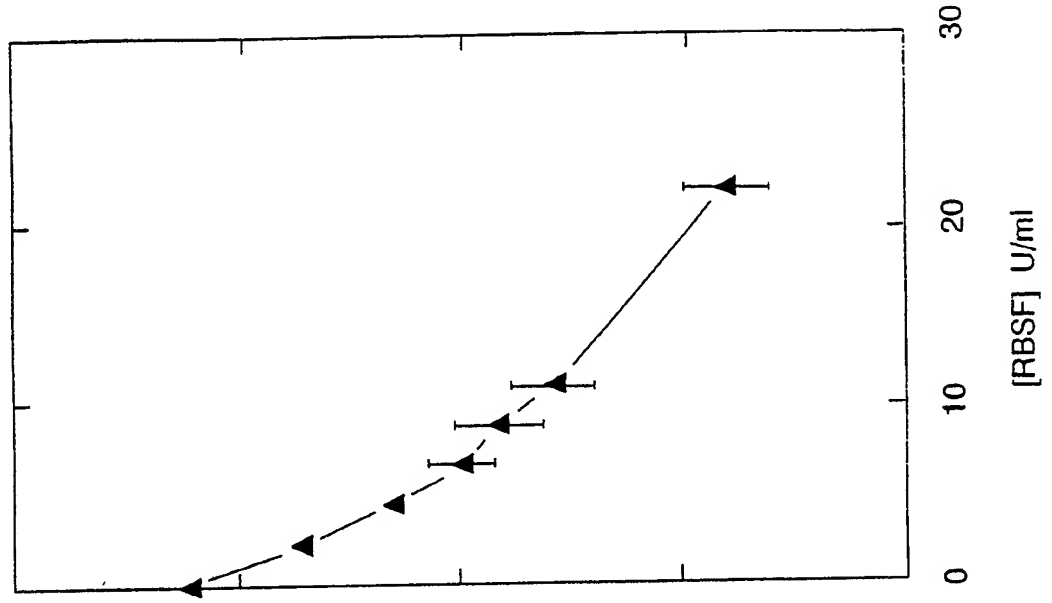

30
20
10
0
[RBSF] U/ml

FIG. 7A

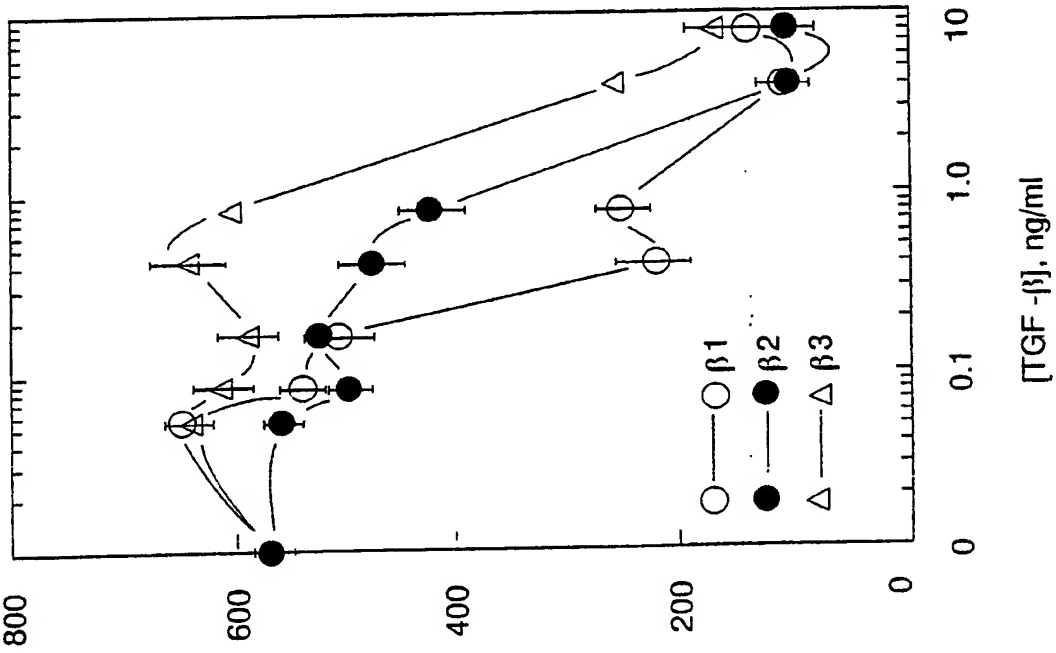

10
1.0
0.1
0
[TGF -β], ng/ml
β1
β2
β3
800
600
400
200
0
H2 O2 (nmol/mg cell protein)

FIG. 8

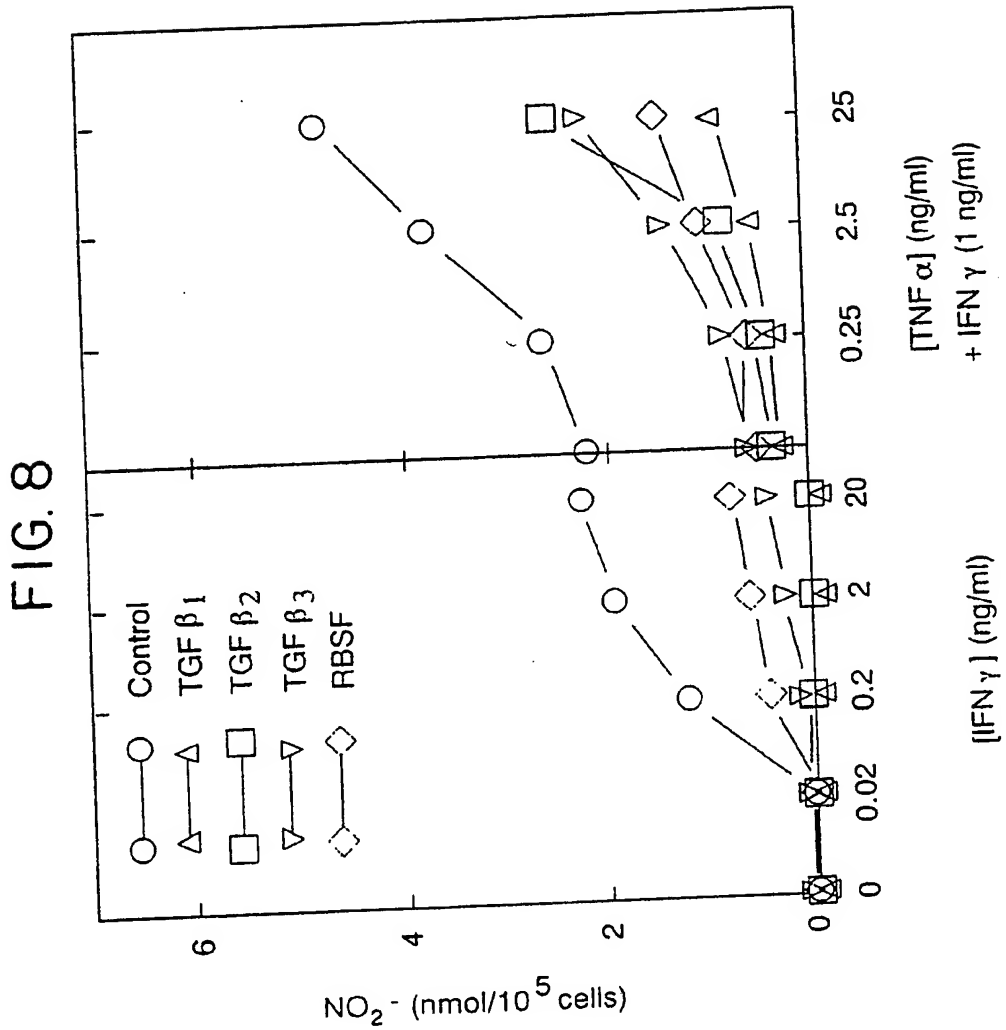

Control
TGF β1
TGF β2
TGF β3
RBSF
0
2
4
6
NO2- (nmol/10^5 cells)
0
0.02
0.2
2
20
[IFN γ] (ng/ml)
0.25
2.5
25
[TNF α] (ng/ml)
+ IFN γ (1 ng/ml)

FIG. 9

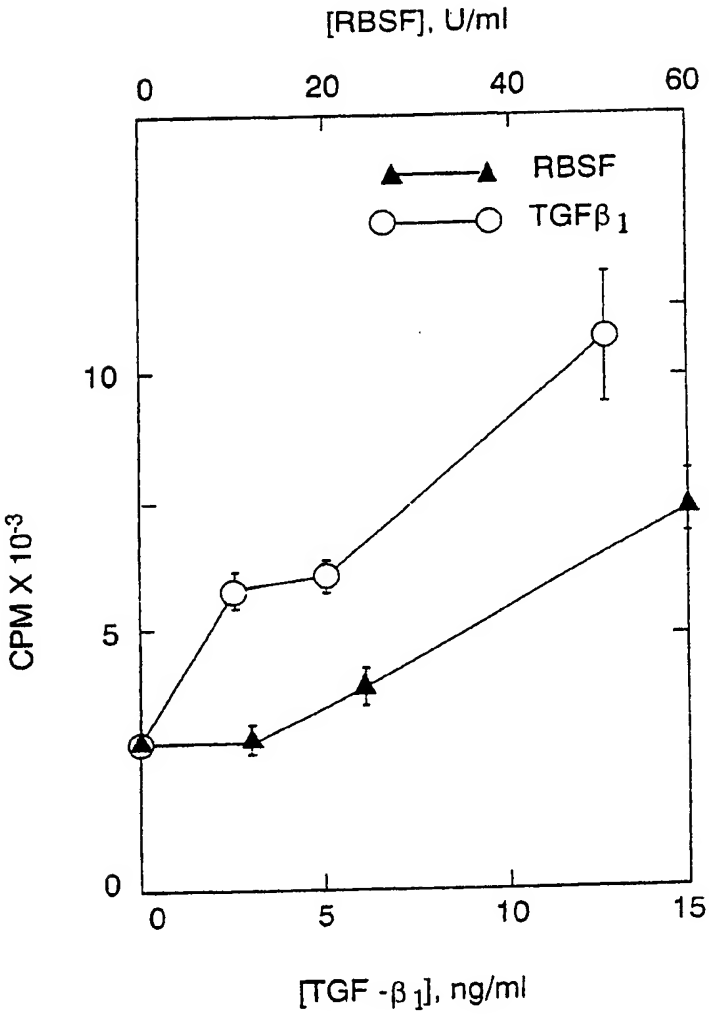
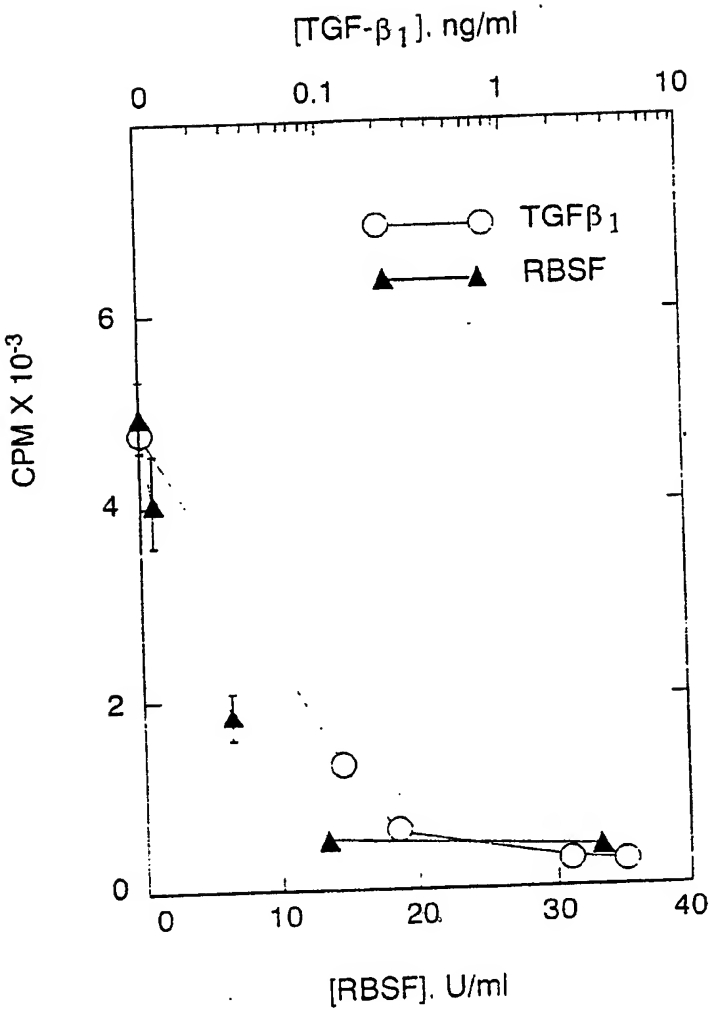


FIG. 10


[TGF-β1], ng/ml
0
0.1
1
10
TGFβ1
RBSF
CPM X 10-3
6
4
2
0
0
10
20
30
40
[RBSF], U/ml

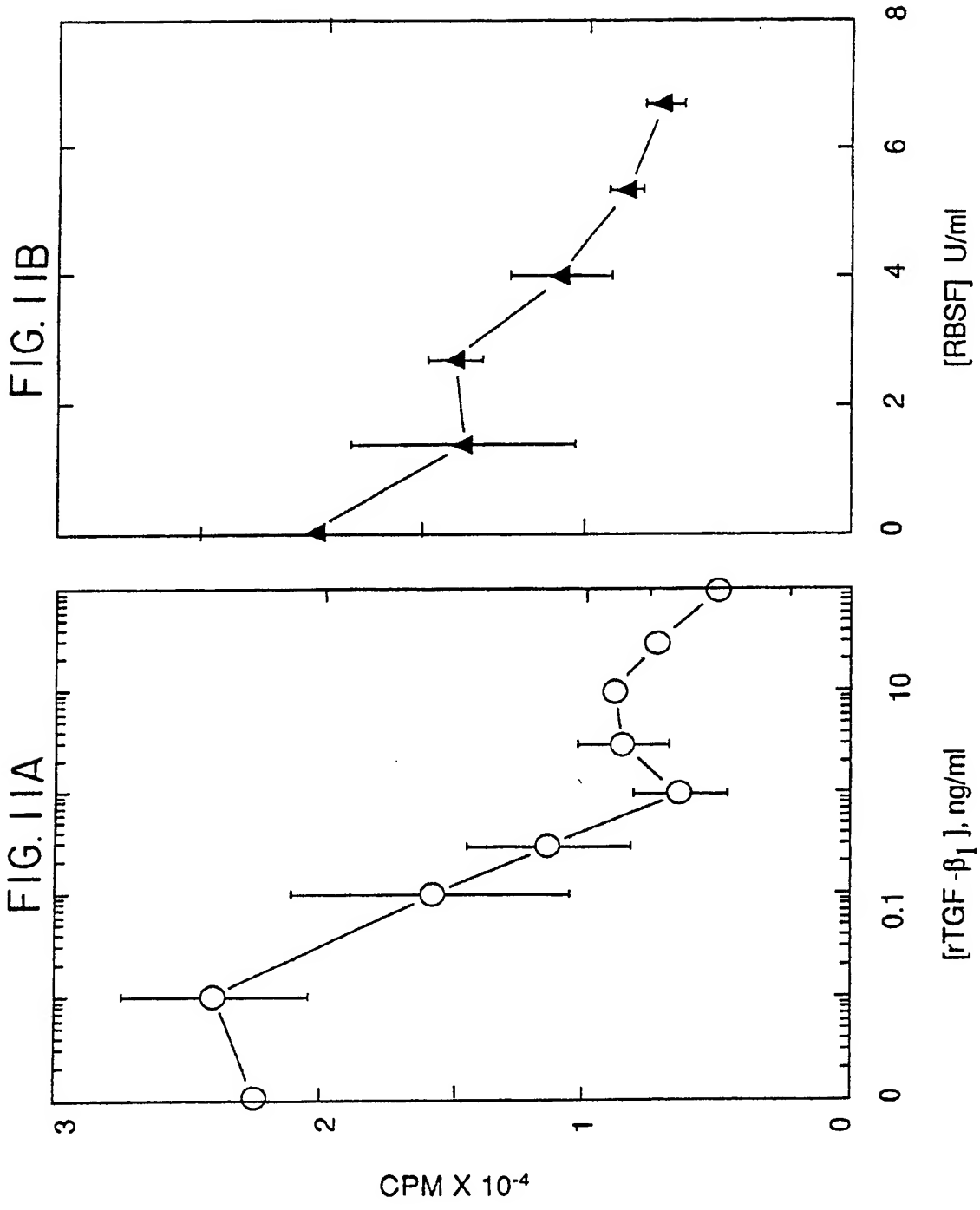


FIG. IIB
FIG. IIA
0
2
4
6
8
[RBSF] U/ml
0
0.1
10
[rTGF -β1], ng/ml
0
1
2
3
CPM X 10-4

FIG. 12A

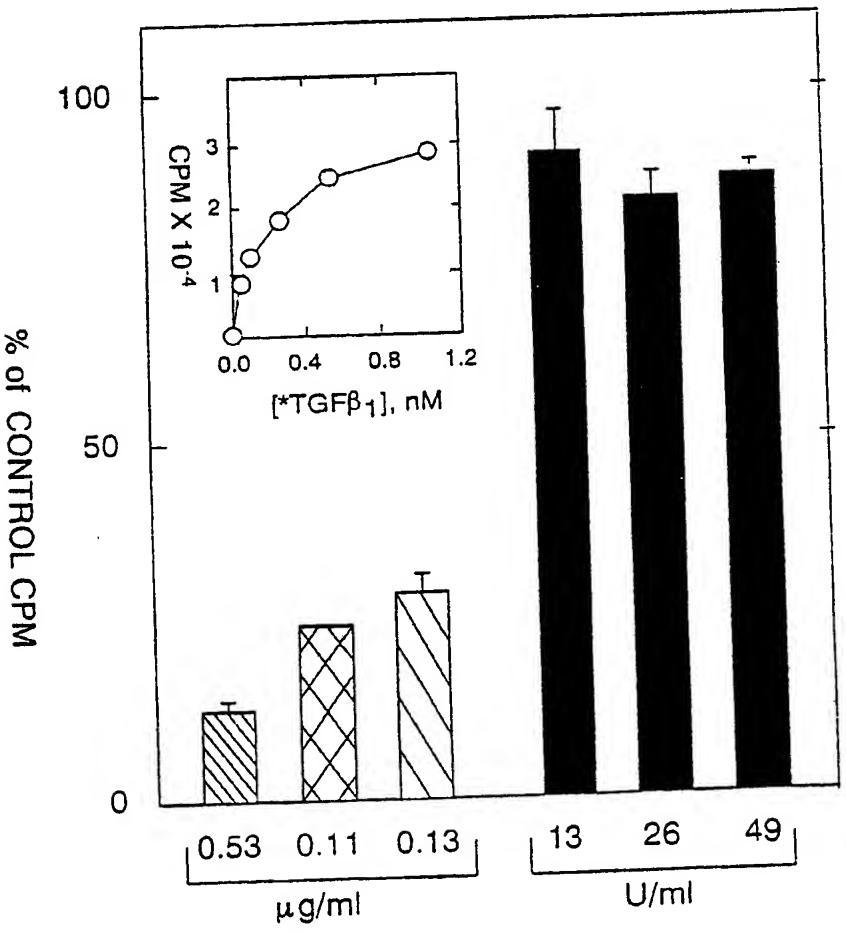

100
50
0
% of CONTROL CPM
CPM X 10⁻⁴
3
2
1
0.0
0.4
0.8
1.2
[*TGFβ1], nM
0.53
0.11
0.13
μg/ml
13
26
49
U/ml

FIG. 12B

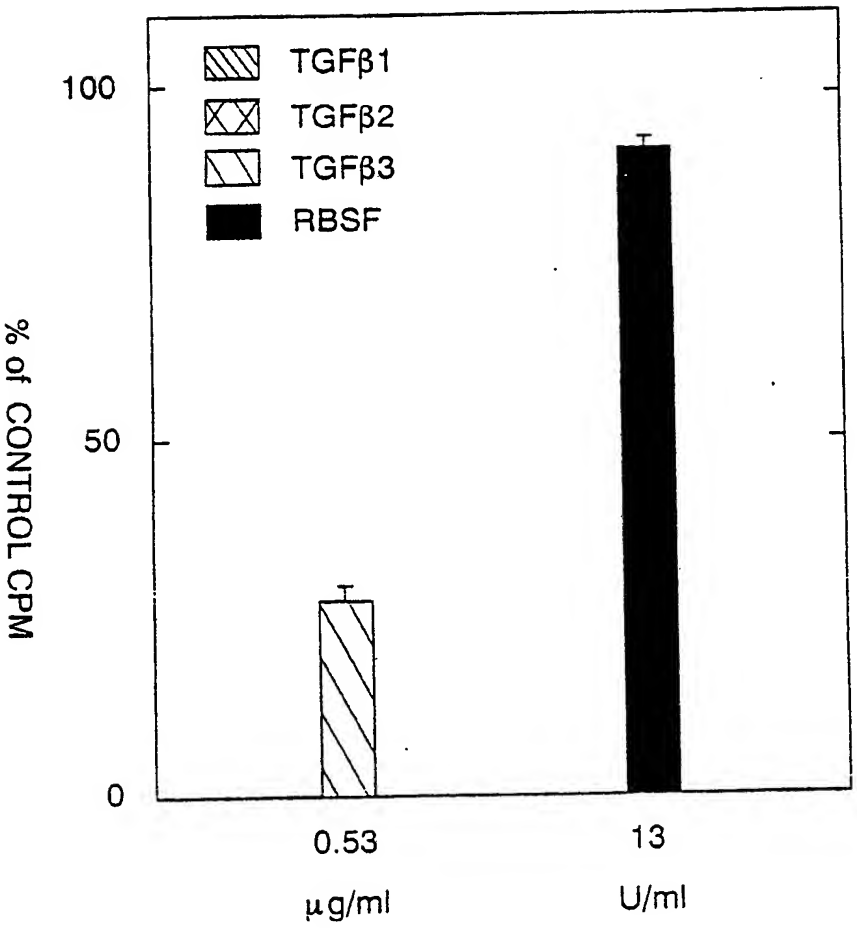
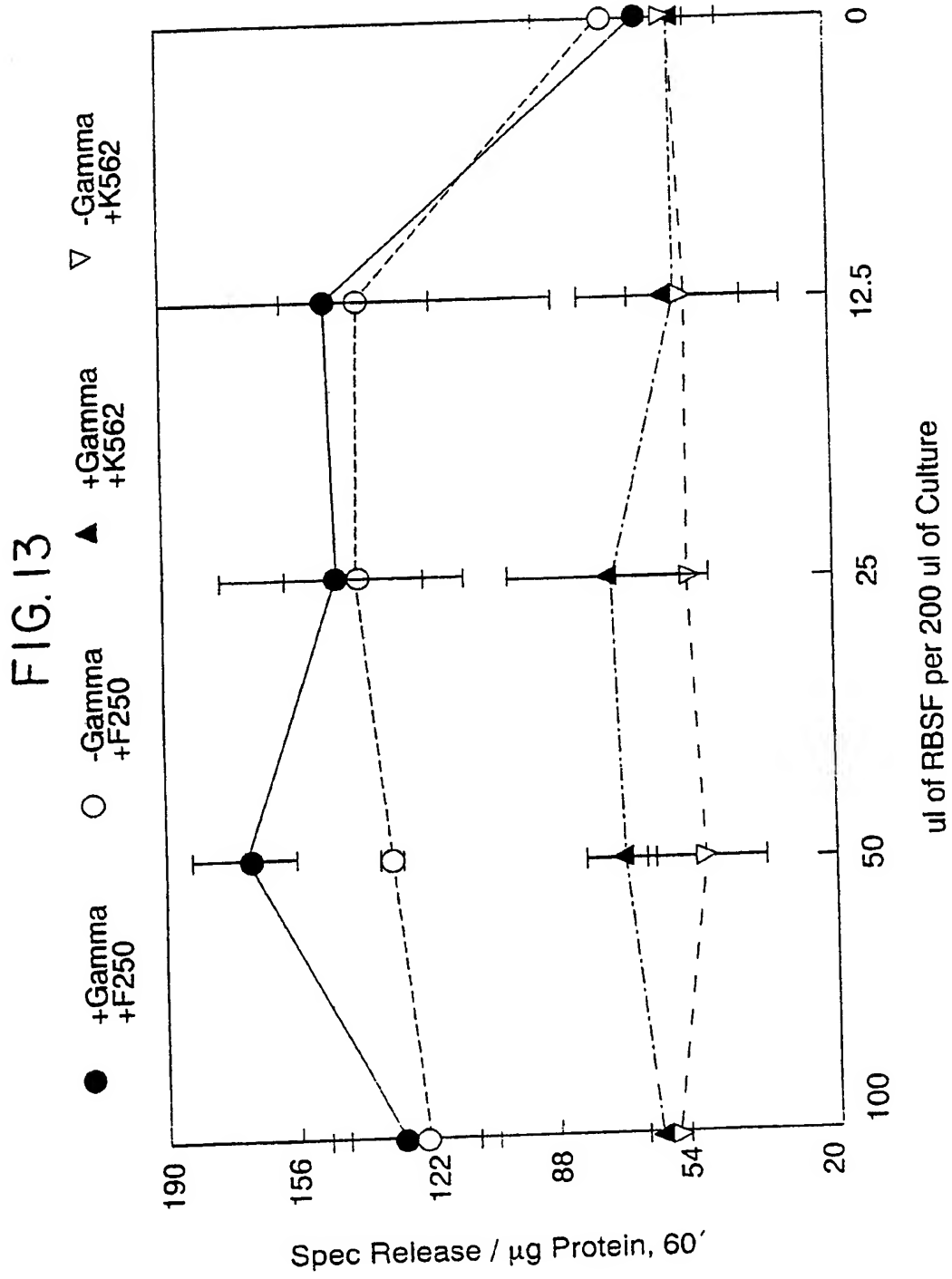


FIG. 13


+Gamma +F250
-Gamma +F250
+Gamma +K562
-Gamma +K562
190
156
122
88
54
20
0
12.5
25
50
100
Spec Release / µg Protein, 60'
ul of RBSF per 200 ul of Culture

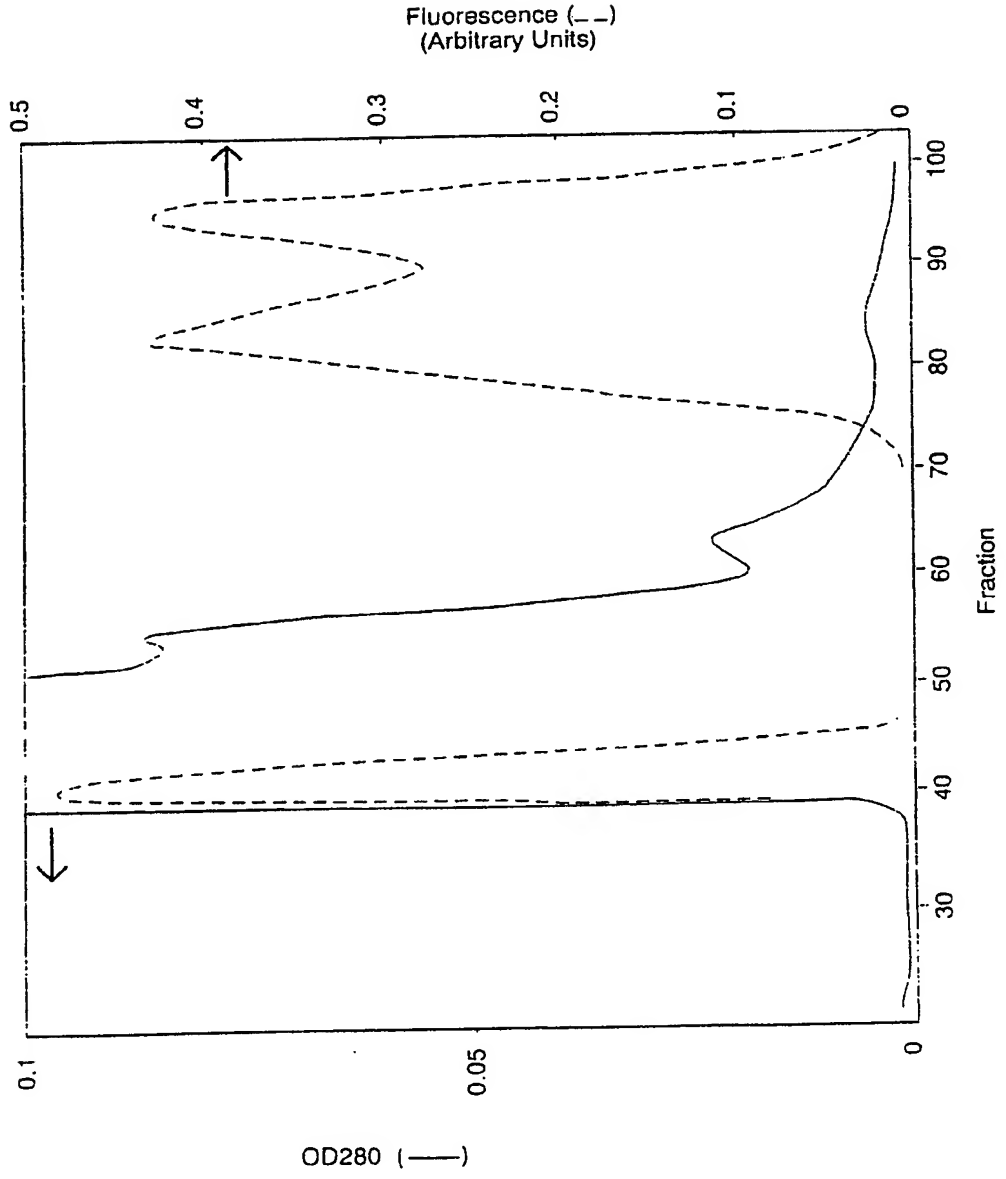


FIG. 14
BIOGEL P60 CHROMATOGRAPHY

FIG. 15

SDS-PAGE OF BIOGEL P60 FRACTIONS

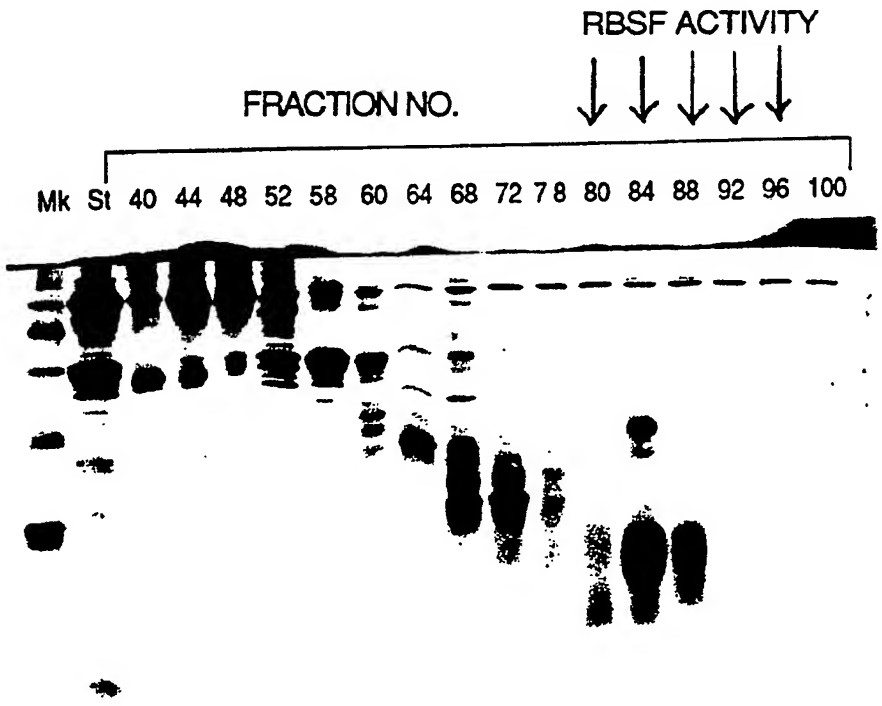


FIG. 16

SP-5PW CHROMATOGRAPHY

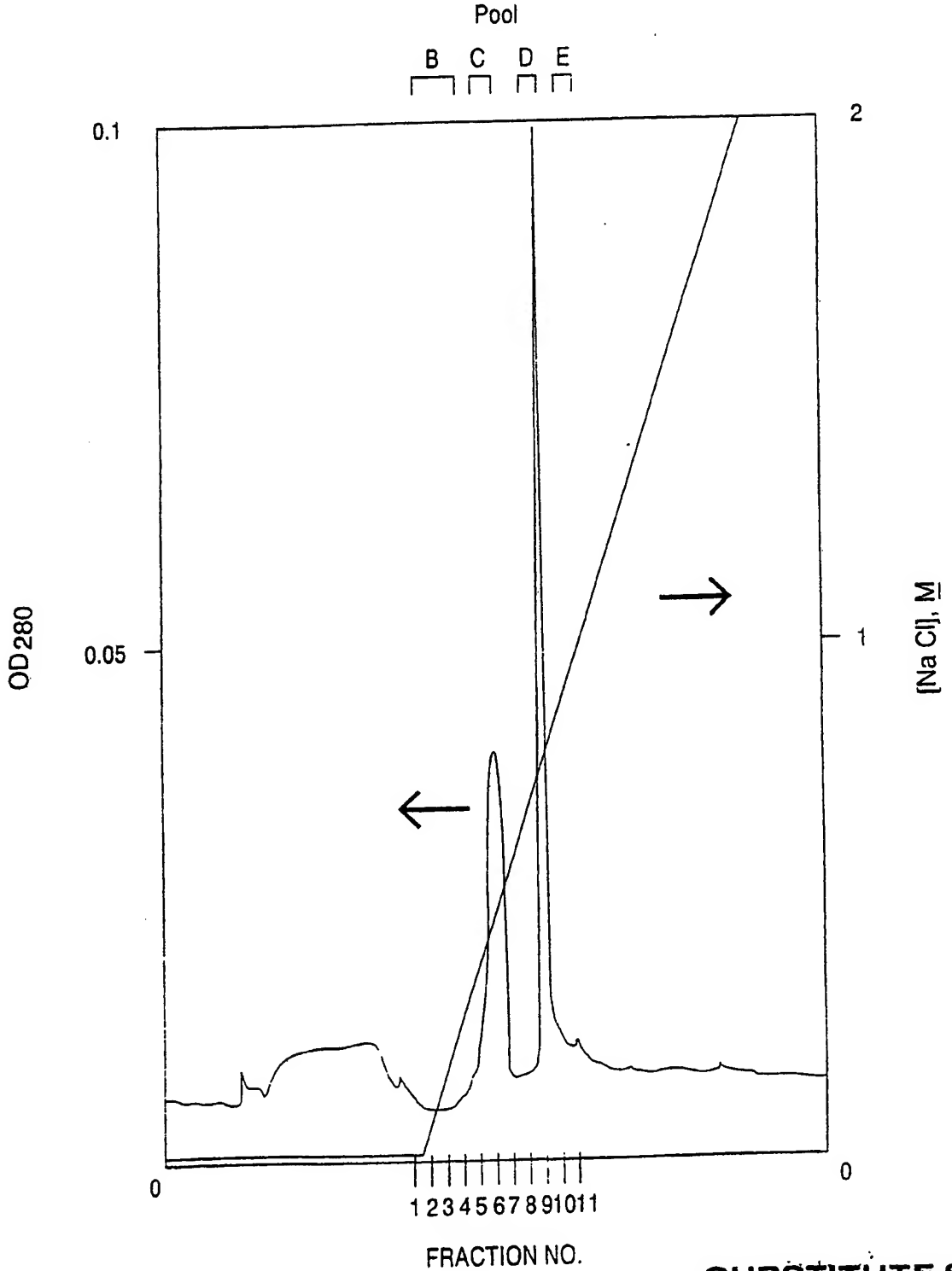
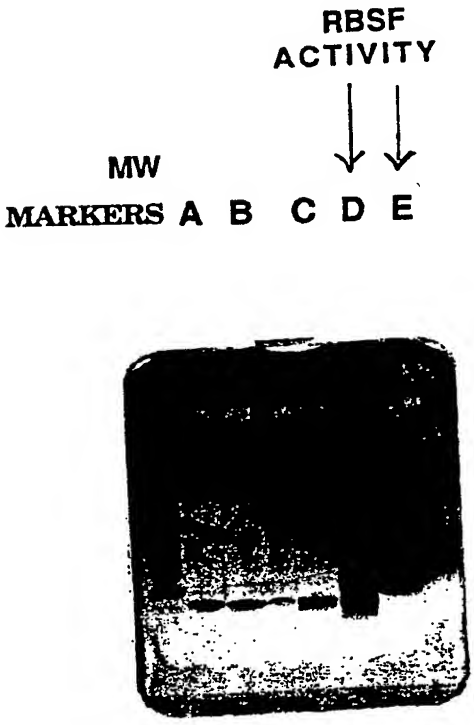


FIG. 17

SDS-PAGE OF SP-5PW FRACTIONS



INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/02136

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 37/02; C07K 15/00, 15/04, 15/28

U.S CL: 514/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S CL:	514/12; 530/324, 350, 351, 387

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	WO, A, 90/00900 (Nathan et al) 08 February 1990 see page 2, line 27-page 3 line 6, page 4. lines 14-20, page 5, lines 30-33. page 6, lines 3-12, page 8, lines 28-33.	1,3,4,8, 14,15-26 6
Y	Wensel et. al., "Radioimmunoimaging and Radioimmunotherapy, Bifunctional chelating agents for binding metal ions to proteins" published 1983 by Elsevier Science Publishing Co., Inc. (NY), see page 185.	6
X Y	J. Immunology, vol. 142, no. 10, 15 May 1989. Tsunawaki et al.. "Comparison of Transforming Growth Factor-B and a Macrophage-Deactivating polypeptide from tumor cells," pages 3462-68, see the abstract, page 3463 column 1.	1-5,7,9,11, 13, 15-26 6, 14
X	US, A, 4,816,561 (Tadoro) 28 March 1989, See column 8. line 68-column 9. line 2. ~~column 13, line 49-column 14, line 13.~~	9, 10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

20 May 1991

Date of Mailing of this International Search Report

21 JUN 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Jeffrey E. Russel

Form PCT/ISA/210 (second sheet) (July 1989)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X/Y	US, A, 4, 530,838 (Evans et al) 23 July 1985, see column 9, lines 3-5, claim 1.	1,3,8,14/6
Y	US, A 4,350,764 (Baxter et al) 21 September 1982, see column 3, lines 9-26.	1,3,6-8 14
Y	J. Immunology vol. 138, no. 11 02 June 1987, Peterson et al., "Opioid -Mediated Suppression of Cultured Peripheral Blood Mononuclear Cell Respiratory Burst Activity", pages 3907-12, see the abstract.	1,3,6-8. 14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	J. Surgical Oncology, vol. 23, issued 1983, Korzelius et al., "Suppressor Substance Produced by the K 562 Cell Line In vitro", see pages 16-20.	11, 12